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The Journal of LABORATORY and CLINICAL MEDICINE

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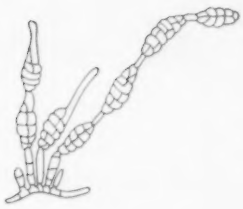

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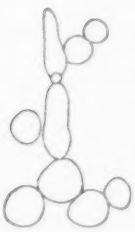

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Source—Free, H. M.; Cook, M. H., and Smeby, R. R.: Comparative Study of Qualitative Tests for Urine Ketones. Presented at 132nd National Meet., Am. Chem. Soc., New York, N. Y., Sept. 8-13, 1957. (Abstracts, p. 78 c).

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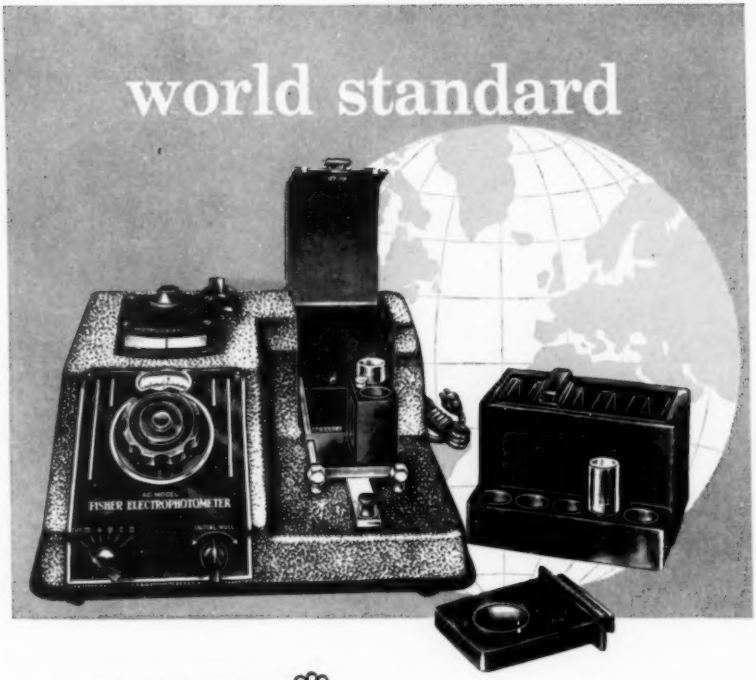

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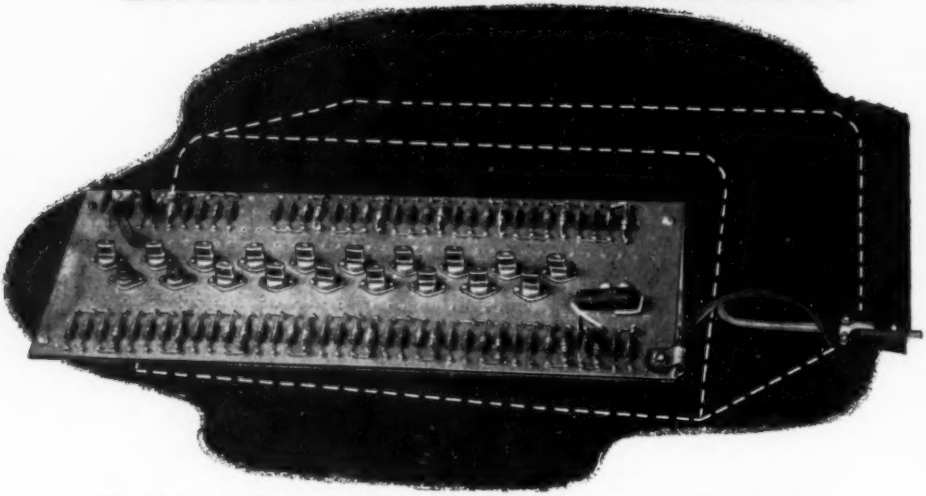

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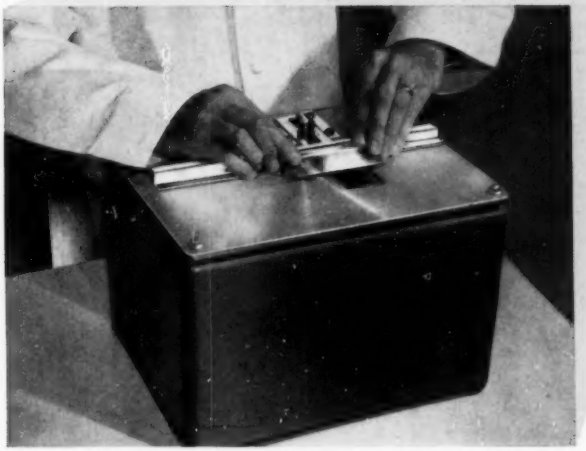

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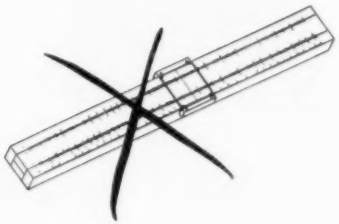

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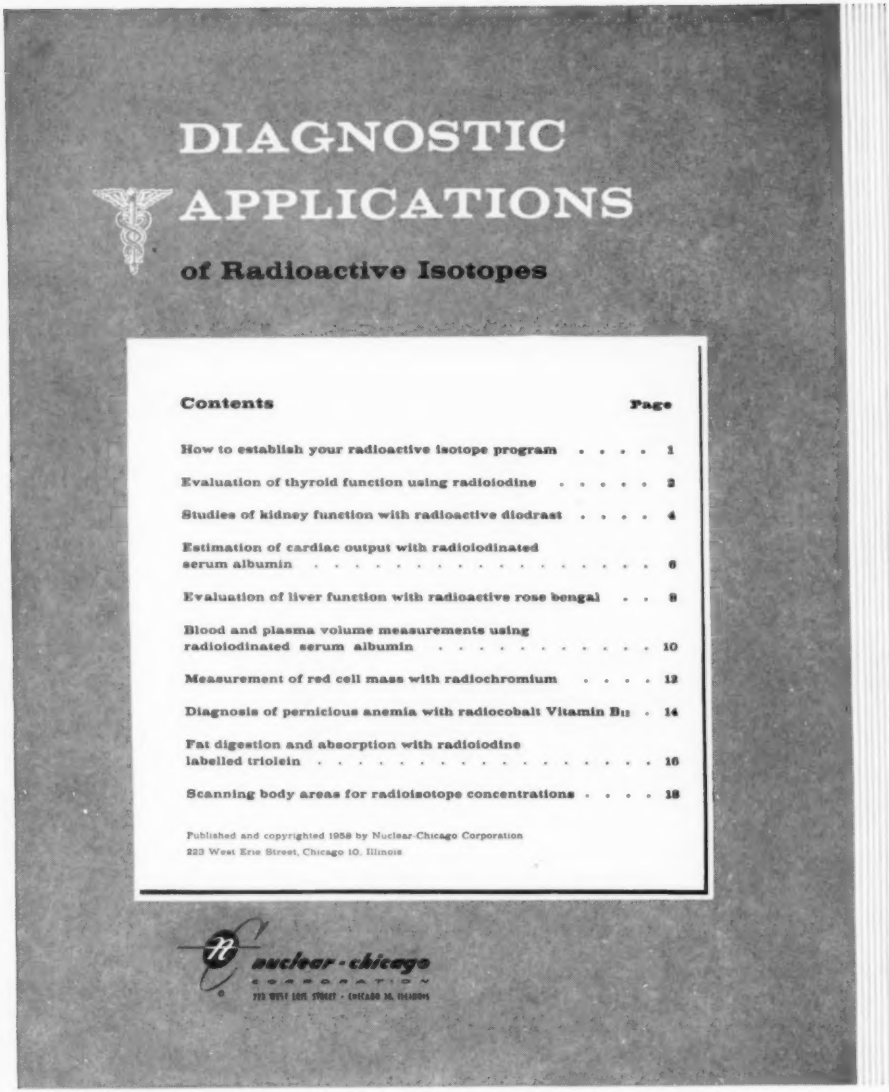
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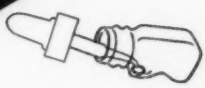

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*Kent, J. F. et al. "Differentiation of the Antilipids Occurring in Non-treponemal Diseases and Syphilis." Jour. Chronic Diseases, Vol. 7, 36-42, 1958.

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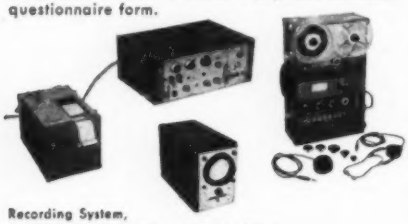

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(1) Bunch, L. D.: Am. J. Clin. Path. 28: 111, 1957.
(2) Wheelock, M. C.: Am. J. Dig. Dis. 3: 178, 1958.

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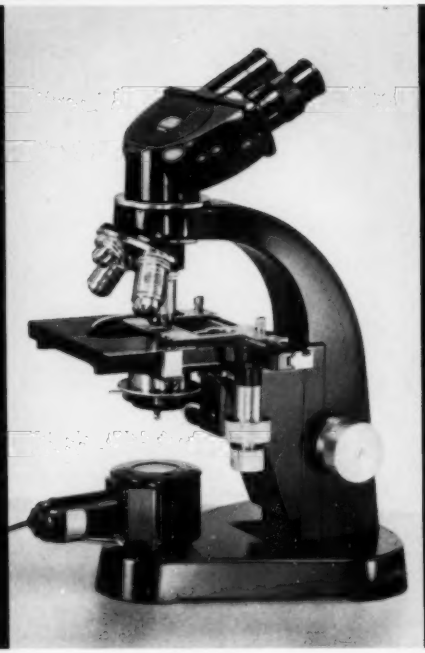

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An Editorial

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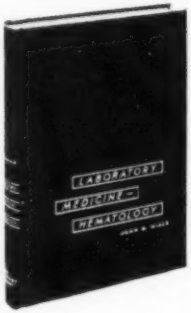

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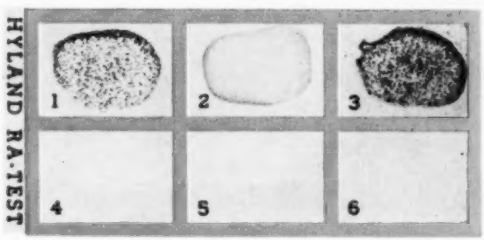

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by

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City of Detroit Receiving Hospital

1956, 164 pages (8½" × 11"), illustrated
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D.
Disorders of Rate and Rhythm.
* * *
E.
Summary of QRS-T Patterns in Unipolar Thoracic and Limb Leads.

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The analysis of the arrhythmias is made from leads that clearly display auricular as well as ventricular deflections; the interpretation of the QRS-T complex is based upon the findings in multiple precordial and back leads, supplemented, when necessary, by esophageal and unipolar limb leads. The registration of the normal and abnormal QRS-T complex is based upon concepts developed by Frank N. Wilson and associates and shown to be consistent with experimental and post-mortem findings. These concepts have been presented in detail and suitably illustrated with diagrams as an introduction to clinical analysis. Efforts have been concentrated on a systematic lucid description of electrocardiographic findings in an attempt to make reproduction of tracings unnecessary.

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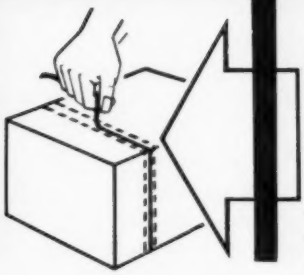

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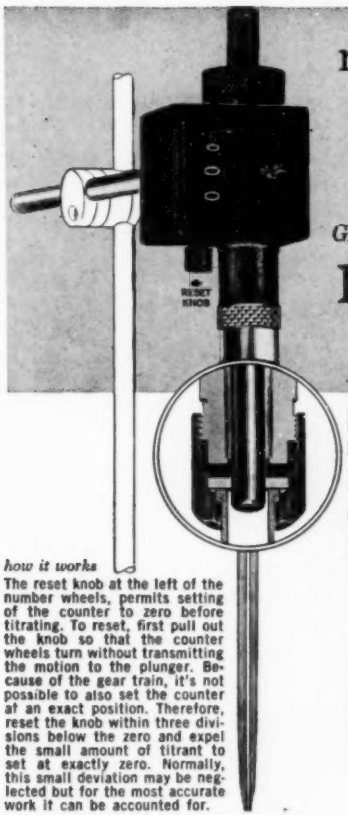

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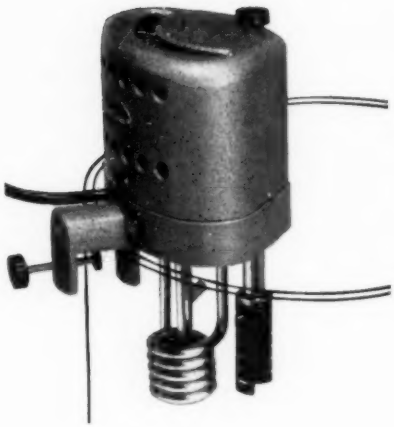

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VOL. 51 JUNE, 1958 NO. 6

CLINICAL AND EXPERIMENTAL

THE DYE DILUTION CURVE IN THE EVALUATION OF MITRAL INSUFFICIENCY

THEODORE F. HUBBARD, M.D.
OMAHA, NEB.

SINCE the introduction of dye dilution curves for the study of cardiac and vascular shunts it has been observed that major degrees of valvular insufficiency cause changes in the curve similar to those due to central circulatory left-to-right shunts. Characteristically there is prolongation of the disappearance time and build-up time of the curve with relative shortening of the appearance time, decrease in the peak concentration of the dye, and, if the insufficiency is of major degree, a disappearance of the recirculation peak. The ratio of the disappearance time to either the appearance time or build-up time is prolonged. These effects are observed when the indicator is injected into the chamber just distal to the incompetent valve or at any point proximal to the incompetent valve and the curve recorded at any point downstream to the incompetent valve. In the absence of valvular insufficiency or central circulatory shunts all the above parameters of the curve are determined by the cardiac output and the central blood volume. The latter must be known or their effects otherwise corrected for in the assessment of the changes due to valvular insufficiency.

Korner and Shillingford,[1] studying the form of the dye dilution curve by means of a cardiocirculatory model, found that in the absence of valve incompetence the cardiac output and central blood volume predicted the disappearance slope of the dye curve with a relatively small error. Empirical formulas were derived from such data which allowed a quantitative estimate of the degree of

From the Bishop Clarkson Memorial Hospital and The University of Nebraska College of Medicine, Omaha, Neb.

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Received for publication Nov. 11, 1957.

valvular insufficiency based upon the observed decrease of the slope of the disappearance limb below that predicted from the cardiac output and central blood volume.

Recently Woodward, Ellis, and Wood[2] have evaluated 12 parameters of the dye dilution curve in the detection and estimation of the degree of valvular insufficiency and have found several of these useful in the differential diagnosis of mitral stenosis and mitral insufficiency.

It was the purpose of the present study to evaluate the dye dilution method for the detection and quantitative estimation of mitral insufficiency. We have studied the oximetrically recorded dye dilution curve obtained after intravenous injection of Evans blue dye in 66 patients with mitral insufficiency. These have been compared with curves obtained from 22 patients with pure mitral stenosis and 20 normal individuals.

METHOD AND MATERIALS

The instrument used for obtaining the dye dilution curves was a Wood type earpiece oximeter. The output of the earpiece photo-cells was electronically amplified by means of a contact modulated D.C. amplifier and recorded on a commercial, direct-writing 10 in. strip chart recorder. All curves used in this series were recorded from the ear after the rapid injection of 20 to 40 mg. of Evans blue dye* into an antecubital vein. The nomenclature used in describing the parameters of the curve is that shown in Fig. 1.

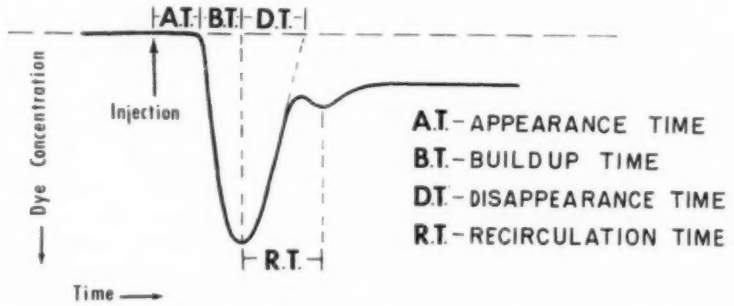

Fig. 1.—Nomenclature used in describing the parameters of the dye dilution curve.

In preliminary studies we had found that the most useful parameter of the curve in the assessment of mitral insufficiency was the ratio of the disappearance time (D.T.) to the appearance time (A.T.), and the evaluation of this parameter forms the basis of the present study. Since the disappearance time could be predicted from the appearance time with a small error in the normal individuals and patients with pure mitral stenosis, the Korner-Shillingford relationship was applied to the data according to the following adaptation:

$$\text{Regurgitant flow (per cent of aortic flow)} = \left(\frac{\text{D.T./A.T. (observed)}}{\text{D.T./A.T. (predicted)}} - 1 \right) \times 100$$

In 31 of the patients with mitral insufficiency the cardiac output and central blood volume determined from the dye curves were used to calculate the volume of regurgitant flow according to the original Korner-Shillingford relationship:

*The Evans blue dye in concentration of 40 mg. per cubic centimeter was kindly supplied by the Warner-Chilcott Laboratories, New York, N. Y.

$$\log 1/s = 0.8799 - 0.061 \text{ C.O.} + 1.112 \log V$$

where log 1/s is the reciprocal of the slope of the disappearance limb of the curve $\left(1/s = \frac{t_2 - t_1}{\log_e C_1 - \log_e C_2}\right)$; C.O. is the total left ventricular output in liters per minute; and V is the central blood volume in cubic centimeters determined from the dye curve by the method of Hamilton and associates.[3] The above equation is solved for C.O. and the volume of phasic backflow, B, determined from the following: B = C.O. - F.F. where F.F. is the aortic flow in liters per minute determined from the dye curve by the Hamilton method. The regurgitant flow determined by the latter was compared to that obtained by the present method.

The subjects of the study consisted of 5 groups of patients. (1) Twenty normal individuals; (2) 22 patients with pure mitral stenosis: in each of these patients the absence of mitral insufficiency was established at the time of mitral commissurotomy; (3) 17 patients with mitral insufficiency grade 1: the presence of a minimal degree of mitral insufficiency was established in each of these patients at the time of mitral commissurotomy; all the patients in this group had varying degrees of mitral stenosis as their predominant dynamic lesion. (4) Thirty-two patients with mitral insufficiency grade 2: the presence of a moderate degree of mitral insufficiency was established in 15 patients of this group at the time of mitral valve surgery; in the remaining cases the estimation of the degree of mitral insufficiency was based upon the results of clinical evaluation and, in some of the cases, left heart catheterization. (5) Seventeen patients with mitral insufficiency grade 3: none of these patients was operated upon but all were considered on the basis of clinical evaluation, autopsy, or left heart catheterization to have severe mitral insufficiency; a few of these were considered to have minimal associated mitral stenosis though most were thought to have pure mitral insufficiency.

TABLE I. THE RATIO OF THE DISAPPEARANCE TIME TO THE APPEARANCE TIME OF THE DYE DILUTION CURVE IN NORMAL INDIVIDUALS AND PATIENTS WITH MITRAL STENOSIS AND THREE GRADES OF MITRAL INSUFFICIENCY

	NORMAL	MITRAL STENOSIS	MITRAL INSUFFICIENCY GRADE 1	MITRAL INSUFFICIENCY GRADE 2	MITRAL INSUFFICIENCY GRADE 3
No. of patients	20	22	17	32	17
D.T./A.T.* ± S.D.	1.2 ± 0.3	1.2 ± 0.16	1.5 ± 0.55	2.0 ± 0.45	5.8 ± 2.2
Range	0.8 - 1.9	0.8 - 1.6	1.2 - 2.5	1.3 - 3.2	2.5 - 11.0
Regurgitant flow (%)†					
Mean	0	0	20	67	383
Range			0 - 105	8 - 166	108 - 818

*Ratio of disappearance time to appearance time ± standard deviation.
†Calculated according to the following:

$$\text{R.F. (\% of aortic flow)} = \left(\frac{\text{D.T./A.T.}}{1.2} - 1\right) \times 100.$$

RESULTS

Table I summarizes the results of this study. The dye dilution curves obtained from the normal control group showed a mean disappearance time–appearance time ratio (D.T./A.T.) of 1.2 ± 0.3 (standard deviation). The mean D.T./A.T. in the group of patients with pure mitral stenosis was 1.2 ± 0.16 and did not differ significantly from the control group. The mean D.T./A.T. in the group of patients with mitral insufficiency grade 1 was 1.5 ± 0.55 and was significantly higher than the means of the control and mitral stenosis groups ($P = < 0.05$). The groups with mitral insufficiency grade 2 and mitral insufficiency grade 3 had respective mean D.T./A.T. values of 2.0 ± 0.45 and 5.8 ± 2.2. The values for these groups differed significantly from each other ($P = < 0.02$) and from the previous groups.

Fig. 2 is a scattergram of the D.T./A.T. values in the different groups. It can be seen that there is considerable overlap of the values between the subjects with mitral insufficiency grade 1 and the control and mitral stenosis groups. There was small overlap between the values in the latter groups and those of mitral insufficiency grade 2, and there was no overlap between the group with mitral insufficiency grade 3 and the normal and mitral stenosis groups.

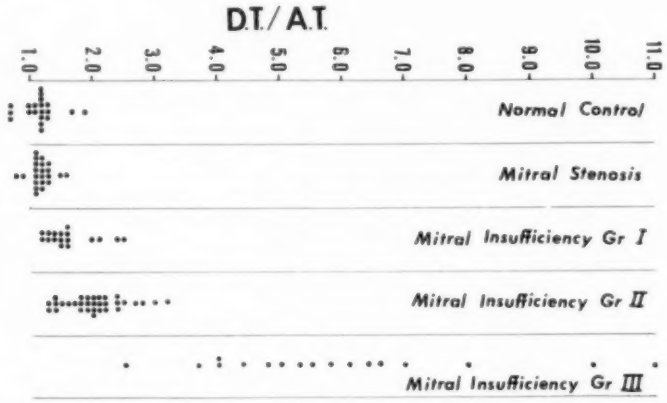

Fig. 2.—Scattergram showing the ratios of disappearance time to build-up time in the 5 groups in this study.

It was observed in the control and mitral stenosis groups that the appearance time of the dye dilution curve predicted the disappearance time (D.T. = 1.2 × A.T.) with a standard error of 0.05. The estimation of the degree of mitral insufficiency was thus calculated from the D.T./A.T. value by application of the relationship derived from the hydraulic data of Korner and Shillingford:

$$\text{Regurgitant flow (per cent of aortic flow)} = \left(\frac{\text{Observed D.T./A.T.}}{1.2} - 1\right) \times 100$$

Applying this relationship it was found that in the patients with clinical mitral insufficiency grade 1 the mean regurgitant flow was equal to 20 per cent of the aortic flow with a range of 0 to 105 per cent. In mitral insufficiency grade 2 the mean regurgitant flow was equal to 67 per cent of the aortic flow with a range of 8 to 166 per cent. In mitral insufficiency grade 3, the mean regurgitant flow was 383 per cent of the aortic flow with a range of from 108 to 818 per cent.

Fig. 3 shows the relation of the regurgitant flow calculated by the present method to the regurgitant flow calculated by the original method of Korner and Shillingford. There is generally good agreement between the two methods (coefficient of correlation = 0.9) although the present method was apparently more sensitive in detecting slight degrees of valvular insufficiency.

Fig. 4 shows some representative dye curves from this study.

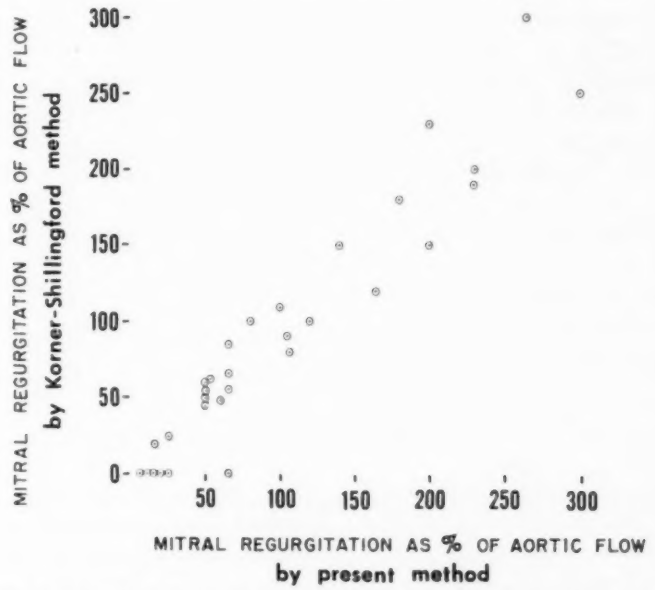

Fig. 3.—The relationship of the relative volume of regurgitation determined by the present method and the Korner and Shillingford method.

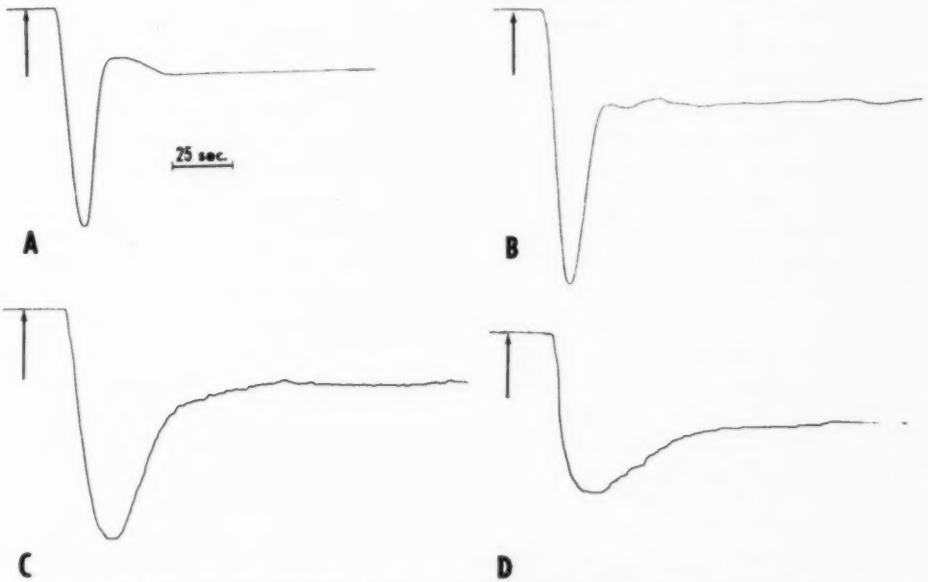

Fig. 4.—Some representative dye dilution curves from this study. A, Normal subject; D.T./A.T. = 1.0 B, Pure mitral stenosis; D.T./A.T. = 1.3. C, Clinical mitral insufficiency grade 2; D.T./A.T. = 2.1, the estimated regurgitant flow is 75 per cent of aortic flow. D, Clinical pure mitral insufficiency grade 3; D.T./A.T. = 5.2, the estimated regurgitant flow is 330 per cent of aortic flow.

DISCUSSION

There is need for a quantitative technique for the estimation of valvular insufficiency both in clinical valvular heart disease and in the study of the physiology of valvular regurgitation. There being no absolute standard for determining the accuracy and reliability of the present method, we have had to depend upon comparisons between the dye method and assessments of insufficiency by the surgeon or by clinical methods. With the method used herein there is a statistically significant correlation between the D.T./A.T. ratio of the dye curve and the surgical or clinical estimate of the degree of mitral insufficiency. Although there was a statistically significant difference between the mean D.T./A.T. in the normal or pure mitral stenosis groups and the group with minimal mitral insufficiency there was considerable overlap of values. Thus in the individual patient the method would poorly discriminate between minimal insufficiency and no insufficiency.

The principles evolved by Korner and Shillingford from studies of the dye curve in valvular insufficiency in hydraulic models would seem to have a sound physical basis. Using the method of Korner and Shillingford as a standard for determination of the quantitative accuracy of the present method the two methods show good correlation, although the latter was more sensitive to minimal insufficiency. The volumes of regurgitant flow as compared to aortic flow obtained by the present method generally seem plausible and are comparable to the values obtained by Novack and associates[4] using the Korner and Shillingford method in patients with mitral disease. Although some of the values obtained in the group with mitral insufficiency grade 3 are very high, they are in agreement with the highest values obtained by Woodward, Ellis, and Wood[2] and by Milnor[5] in experimental mitral insufficiency in dogs. However, it is possible that, in the highest grades of mitral insufficiency, systemically recirculated dye may influence the estimation of the disappearance time of the primary curve.

Recently the character of the left atrial pressure waves as determined by left heart catheterization has been used for the assessment of mitral insufficiency. The estimate is based predominantly on the form and magnitude of the left atrial V wave. Although there was fairly good agreement between the left heart catheterization data and the estimation of mitral insufficiency by the present method, there were too few such comparisons for reliability.

The present study was restricted to patients with mitral valve disease who had no evidence of other valvular lesions. However, injection of the dye into an arm vein with recording of the curve from a peripheral site measures the total insufficiency from all valve sites. The technique can be applied differentially by intracardiac injection at various sites; the changes of insufficiency will be present in the peripherally recorded curve when the dye is injected at any point proximal to an incompetent valve or into the chamber guarded by an incompetent valve and will be absent when the dye is injected into any chamber distal to the one guarded by an incompetent valve.

Another indicator-dilution method involves the injection of indicator into the chamber just distal to the valve in question and the mathematical comparison of the time concentration curves obtained downstream and just proximal to the valve.[2, 5, 6] Although the theoretical basis of this method is absolute, the practical assumption of rapid and complete mixing of dye with blood under these circumstances would apparently often not obtain.

The present method makes it possible to quickly assess the relative volume of regurgitant flow without the cumbersome mathematical calculations of the Korner and Shillingford method. Such an estimate is thus independent of the absolute abscissal values of the curve and the errors in the calculation of cardiac output and central blood volume. When absolute volume of regurgitant flow is desired, however, it may be readily obtained by determining cardiac output from the curve and converting relative regurgitant flow to absolute flow. Finally, the present method has the virtue of mechanical simplicity and absence of discomfort or danger to the patient.

SUMMARY

The oximetrically recorded dye dilution curve obtained after intravenous injection of Evans blue dye was evaluated as a method of determining the degree of regurgitation in patients with mitral valve disease. Patients were studied with normal hearts, with surgically established pure mitral stenosis, and with mitral insufficiency of 3 grades of severity established by surgery, or by clinical and autopsy studies.

The parameter of the dye curve evaluated in this study was the D.T./A.T. ratio. It was found that mitral stenosis did not alter this ratio, but there was a statistically significant increase in the ratio with increasing degrees of mitral insufficiency. By application of a principle determined by Korner and Shillingford it was found that the 3 groups of mitral insufficiency subjects had respective mean values for regurgitant flow (as per cent of aortic flow) of 20 per cent, 67 per cent, and 383 per cent. Although there is no standard for assessment of the absolute accuracy of the method it appears suitable for the determination of clinical valvular insufficiency.

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STUDIES ON PLATELETS

XX. Further Characterization of the Electrophoretic Anomaly in Serum of Patients With Idiopathic Thrombocytopenic Purpura

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With the Technical Assistance of Joseph Di Masi*** and Lorraine Cassidy

ABNORMALITIES of the protein constituents of serum have been previously described in patients with idiopathic thrombocytopenic purpura (I.T.P.). They may be summarized as follows: (1) protein-bound glucosamine and hexoses are significantly increased[1]; and (2) electrophoretic studies show accumulation of carbohydrates in the albumin and gamma globulin fractions of serum.[2] The abnormal carbohydrate material persists in the albumin fraction of serum even after clinical and hematologic recovery.[2] The present studies were undertaken to clarify the nature and significance of these anomalies.

MATERIALS AND METHODS

Patients studied included 6 cases of I.T.P., 3 of the acute and 3 of the chronic variety.[3] The presence or absence of platelet antibodies (agglutinins and complement-fixing antibodies†) was verified in each instance by the techniques previously described.[4] Serum from normal individuals and from patients with hemophilia and plasma thromboplastin component (PTC) deficiency (hemophilia B, Christmas disease) was also studied.

Electrophoresis of Serum.—Electrophoretic separation of serum fractions was carried out by a minor modification of a standard technique.[5] Barbital buffer was used of pH 8.6 and ionic strength 0.075. Whatman No. 3 filter paper was divided by pencil line into strips 1¼ in. wide by 13 in. long. A pencil line was made widthwise at 8½ in. The wanted quantity of serum (0.025 ml. for proteins or 0.05 ml. for glycoproteins) was measured with the use of calibrated micropipettes and applied into the edge of a clean glass slide. This edge was then pressed to the 8½ in. pencil line of the paper, a fairly even distribution being obtained in this manner. Current was passed for 7½ hours at 180 v. (approximately 18 Ma.). At the end of this time, 2 of the strips were cut off and dried in an oven at 50° C. for 20 minutes. All experiments were run in duplicate.

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Electrophoretic Studies of the Albumin Fraction.—One strip was stained immediately for protein by the method of Block, Durrum, and Zweig.[5] The albumin fraction was then located in the unstained strip by apposition of the stained and unstained samples. At the same time, we prepared paper strips of the same size which were then immersed in pH 4.5 phthalate buffer and blotted to remove the excess moisture. The wet, unstained area containing the albumin fraction was then separated by cutting the paper transversely and placed tightly on the new strip. More phthallate buffer was sprayed with an atomizer over the top of the applied section, to assure good contact with the paper below, and pressure was applied with an applicator stick. Electrophoretic migration was continued for 14½ hours at 120 v. (approximately 10 Ma.) using phthalate buffer. All experiments were run in duplicate. One strip was then stained for protein and the other for glycoprotein using the techniques of block and co-workers[5] and Köiw and Grönwall,[6] respectively. Density values were finally read with the use of a photovolt apparatus using a wave length of 570 λ for proteins and 545 λ for glycoproteins. The quantitative values were reported as percentages of total density calculated with a compensatory planimeter.

Elution of Serum Fractions for Chemical Studies.—After migration, the segments of unstained paper strip where albumin and various globulin fractions had been located were transferred separately to glass tubes containing 10 ml. of 0.1 NaOH solution. Elution was carried out at room temperature and was complete in 10 minutes, being facilitated by squeezing the paper with a glass rod.

Eluates were divided into equal fractions, and fragments of filter paper were precipitated by high speed centrifugation. In a first series of experiments, the supernatant fluid was first decanted. Total proteins were determined by the method of Gornwall and associates,[7] hexoses by the tryptophane method of Sheppard and Everett,[8] and glucosamine by the method of Rimington.[9] Because of the need for further control of our findings, a second series of experiments was conducted. Glucosamine determinations were repeated by the method of Boas[10] which is based on the isolation of the compound on Dowex-50 resin columns, after preliminary hydrolysis. This is followed by determination of the glucosamine by a modification of the Elson-Morgan method.[11] Full details of this technique may be found in recent publications.[12, 13] Values reported for glucosamine in the course of this paper are those obtained by the latter procedure. They were in fairly close agreement with those obtained by the alternate technique.

TABLE I. ELECTROPHORETIC DISTRIBUTION OF SERUM PROTEINS AND GLYCOPROTEINS IN I.T.P., HEMOPHILIA SYNDROMES, AND NORMAL INDIVIDUALS

SERA, PATIENT, SEX, AND AGE	PLATELET AGGLUTININS	COMPLEMENT FIXATION*	PROTEIN (PER CENT)					GLYCOPROTEIN (5 PER CENT)				
			ALB.	α_1	α_2	β	γ	ALB.	α_1	α_2	β	γ
Normal (R. M., F., 29)			45.2	4.8	11.4	16.6	22.0	6.4	27.2	32.4	16.8	17.2
Normal (M. S., M., 38)			37.8	6.7	10.3	18.7	26.5	7.6	24.2	37.5	17.5	13.5
I.T.P. (C. T., F., 21)	1:32	+	33.0	9.0	15.0	21.0	22.0	16.4	17.6	27.3	19.2	19.5
I.T.P. (D. C., M., 5)	1:2	0	43.1	5.9	13.0	14.0	24.0	26.0	11.1	14.8	25.9	22.2
I.T.P. (D. H., F., 6)		0	30.4	9.4	16.9	18.8	24.5	21.2	18.4	26.3	18.4	15.7
I.T.P. (A. C., F., 64)	1:28	+	36.4	7.5	10.6	18.0	27.5	22.4	16.6	11.1	22.2	27.7
I.T.P. (A. R., M., 9)		0	37.0	8.0	13.0	17.0	25.0	21.5	21.4	25.0	21.4	10.7
I.T.P. (G. F., M., 35)	1:512	+	28.4	12.3	13.3	20.0	27.0	20.0	25.0	19.1	16.6	19.3
Hemophilia (S. W., M., 42)			30.0	9.4	12.1	20.2	28.3	–	36.3	21.0	5.0	24.0
PTC deficiency (W. P., M., 39)			32.9	10.1	10.9	18.7	27.4	3.4	30.1	24.2	24.2	18.1

*A lyophilized preparation from platelets was used as antigen.

RESULTS

Electrophoretic Patterns of Normal and Pathologic Sera.—Migration with pH 8.6 barbital buffer gave the protein and glycoprotein patterns for normal

and I.T.P. sera which have been described elsewhere.[2] Actual results are summarized in Fig. 1 and Table I. Sera of patients with hemophilia and PTC deficiency also gave a normal pattern. These cases were selected because it

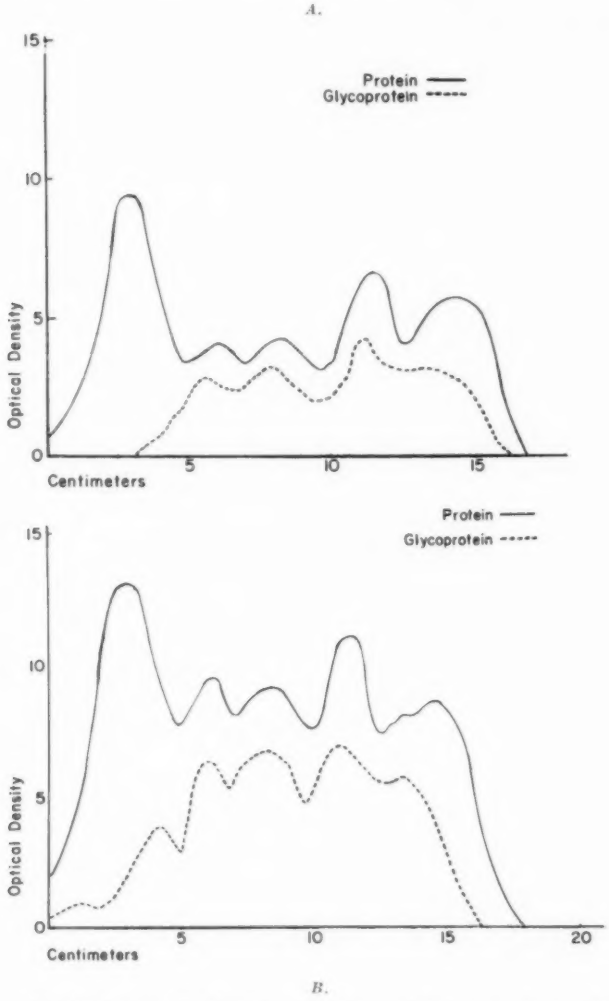

Fig. 1.—Electrophoretic distribution of serum proteins and glycoproteins (semischematic). *A*, Normal serum. *B*, Serum of patients with I.T.P. (note increased amount of carbohydrates in the albumin fraction).

had been previously shown that the standard Tiselius method reveals an abnormal electrophoretic fraction (α_x) in the serum of patients with I.T.P. and with the hemophilia syndrome.[14]

The albumin fraction from normal serum behaved as a single component when electrophoretically migrated in phthalate buffer (Fig. 2, *A*) and showed no appreciable stainable carbohydrate material. The albumin from serum of

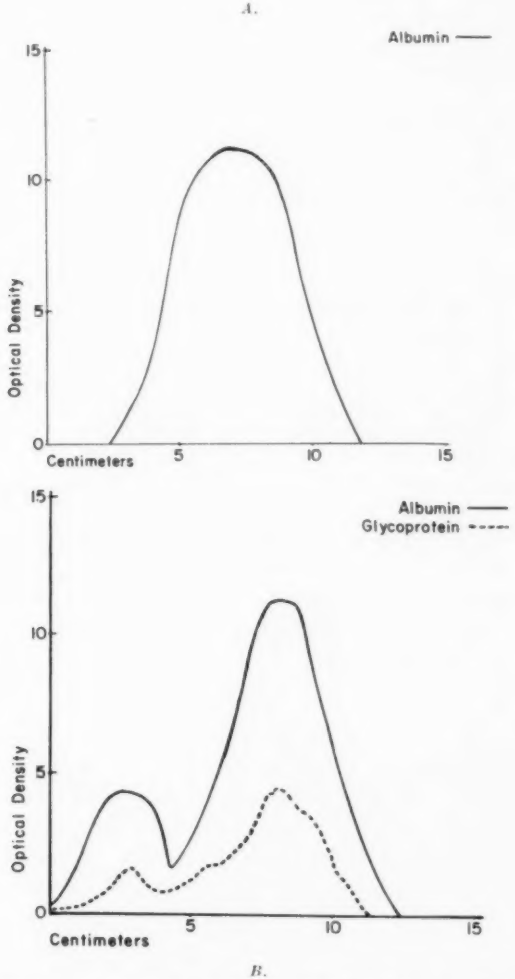

Fig. 2.—Electrophoretic migration of the albumin fraction from sera of normal individuals and patients with I.T.P. in acid buffer (pH 4.6). *A*, Normal serum (note the single component, containing no hexoses). *B*, Serum of patients with I.T.P. (note double component, the faster one containing the greatest amount of hexoses).

patients with I.T.P., however, under identical experimental conditions, separated into 2 individual components: a faster one (albumin-1) representing 24 to 36 per cent of all protein (70 to 85 per cent of the total albumin fraction);

TABLE II. HEXOSES AND GLUCOSAMINE IN THE ELUATES OF VARIOUS ELECTROPHORETIC SERUM PROTEIN FRACTIONS FROM NORMAL AND I.T.P. SERA

SERA, PATIENT, SEX, AND AGE	TYPE	TOTAL		ALBUMIN		α-GLOBULINS		$\beta + \gamma$-GLOBULINS	
		HEXOSES (MG. %)	GLUCOSAMINE (MG. %)	HEXOSES* (MG. %)	GLUCOSAMINE* (MG. %)	HEXOSES (MG. %)	GLUCOSAMINE (MG. %)	HEXOSES (MG. %)	GLUCOSAMINE (MG. %)
Normal Serum		87.0	117	0	0	43.0	61.5	44.0	53.5
Normal Serum		84.2	95	0	0	42.5	51.0	41.7	39.5
Normal Serum		89.2	87	0	0	45.0	49.5	44.2	36.5
I.T.P. (C. T., F., 21)	Chronic	107.0	114	15.0	37.0	51.0	41.0	43.0	33.5
I.T.P. (A. C., F., 64)	Chronic	102.5	138	17.0	34.5	38.5	61.5	47.0	39.5
I.T.P. (G. F., M., 35)	Chronic	97.8	142	16.5	29.0	44.0	53.5	37.3	57.5
I.T.P. (A. R., M., 9)	Acute	100.0	136	17.0	37.5	45.0	54.0	38.0	40.5
I.T.P. (D. C., M., 5)	Acute	100.5	127	18.0	41.5	44.5	43.5	38.0	41.0
I.T.P. (D. H., F., 6)	Acute	102.0	124	16.0	38.0	43.5	52.5	42.5	33.5

*The amounts found in normal subjects were too small for accurate determination.

TABLE III. TOTAL PROTEIN, HEXOSES, AND GLUCOSAMINE CONTENT OF THE TWO ALBUMIN COMPONENTS FOUND IN THE SERUM OF PATIENTS WITH I.T.P.

PATIENT, SEX, AND AGE	TYPE	TOTAL PROTEIN OF SERUM (GM. %)	ALBUMIN-1				ALBUMIN-2			
			PROTEIN		HEXOSES (MG.)	GLUCOSAMINE (MG. %)	PROTEIN		HEXOSES* (MG. %)	GLUCOSAMINE (MG. %)
			GM. %	%			GM. %	%		
I.T.P. (C. T., F., 21)	Chronic	7.6	2.26	29.7	14	32.5	0.25	3.3	0	2.5
I.T.P. (A. C., F., 64)	Chronic	7.5	1.9	25.4	15.5	30	0.82	11	0	4.5
I.T.P. (G. F., M., 35)	Chronic	6.9	1.71	24.8	14.5	27.5	0.25	3.6	0	0
I.T.P. (A. R., M., 9)	Acute	6.5	1.75	27.0	15.5	30.5	0.64	9.9	0	6
I.T.P. (D. C., M., 5)	Acute	7.2	2.6	36.1	16.5	32.5	0.5	7	0	8
I.T.P. (D. H., F., 6)	Acute	7.4	1.91	25.8	15.5	31.5	0.29	3.8	0	6.5

The ratio of bound carbohydrate to protein in terms of milligrams-grams shows an average value of 22.6 for albumin-1 and of 10 for albumin-2.

*Amounts too low for accurate determination.

and a slower one (albumin-2) representing 3.5 to 11 per cent of all protein (Fig. 2, *B*). The bound carbohydrate-protein ratio in terms of milligrams-grams approximated values above 20 for the albumin-1 and of 10 for the albumin-2 fraction. Direct staining with the method of Köiw indicated that 75 to 80 per cent of all hexoses were found in albumin-1 and only 20 to 25 per cent in albumin-2. Similar results were obtained in the serum of rabbits which had developed an auto- and iso- platelet antibody after stimulation with antigenically modified autologous platelets.[15] In two such sera the albumin-1 contained 26 to 27 per cent of all protein, respectively; the albumin-2 between 14 and 17 per cent.

Chemical Studies on the Eluted Electrophoretic Fractions.—The major difference between normal and I.T.P. sera resided in the total amount and in the distribution of hexoses and glucosamine in the various serum fractions. Quantitatively, there was appreciable increase of protein-bound hexoses and a significant rise of glucosamine in the serum of patients with I.T.P. (Table II). Moreover, no appreciable amount of hexoses or glucosamine could be detected in the eluate from the albumin fraction of normal serum, while the eluate from the serum of patients with I.T.P. contained 15 to 20 per cent of the total amount of protein-bound hexoses, and approximately 30 per cent of the total glucosamine (Table II). The increase of total protein bound hexoses and glucosamine in the serum of patients with I.T.P. appeared linked to their appearance in the albumin fraction, while their amount was essentially unchanged in the globulin fractions as compared to normal serum. Table III indicates that hexoses and the greatest part of the glucosamine in the albumin fraction migrated with the albumin-1. A slight amount of glucosamine, however, was recovered from the albumin-2 fraction in the serum of patients with I.T.P. of the so-called "acute variety."

DISCUSSION

The present findings in the serum of patients with I.T.P. confirm and extend the qualitative data reported previously.[2] They essentially indicate for serum of patients with I.T.P.: (1) an absolute increase of protein-bound hexoses and glucosamine; (2) an abnormal presence of protein-bound hexoses and glucosamine in the albumin fraction; and (3) the separation of the albumin fraction in two components (the faster one containing most of the protein, all protein-bound hexoses, and the greatest amount of glucosamine) under certain experimental conditions. The significance of these findings remains uncertain. They are not likely to be the expression of a delayed clotting mechanism, since they do not appear in hemophilic sera, or in sera from patients with secondary thrombocytopenia due to leukemia or bone marrow aplasia. We believe that they may indicate autoimmunization against platelets, especially because similar findings have been shown in the sera of rabbits who had developed auto- and iso- platelet antibodies after stimulation with autologous, antigenically modified platelets.[15] Also, platelet isoagglutinins

were found in the serum of the majority of our patients. We are now extending our studies to additional cases of I.T.P. and to other diseases where an autoimmune factor may play a pathogenetic role (e.g., hemolytic anemias and anaphylactoid purpura) to establish whether the electrophoretic and chemical findings described in I.T.P. apply to all diseases possibly linked to mechanisms of autoimmunization.

The possibility of splitting the albumin fraction and the accumulation of protein-bound hexoses and of glucosamine in the faster moving component of the albumin fraction in the serum of patients with I.T.P. also pose a stimulating challenge of interpretation. The newly observed facts suggest an abnormal composition for the albumin fraction of the serum of patients with I.T.P. We wondered also, about the relationship of these findings to the appearance of the α_x anomaly described by Bernfeld and Stefanini.[14] The two phenomena are obviously unrelated. Thus, hemophilia sera exhibit the α_x anomaly but none of the findings reported here for I.T.P. sera. Finally, the finding of a carbohydrate rich component of the albumin fraction with rapid migration toward the anode at acid pH suggests that this may be represented by orosomucoid, one of the components of the serum glycoproteins. Such a possibility is being explored at present in a larger series of cases.

SUMMARY

1. Electrophoretic studies show an increase of total carbohydrate staining material as compared to normal values and its accumulation in the albumin fraction in the serum of patients with the acute or chronic variety of idiopathic thrombocytopenic purpura (I.T.P.). Chemical studies after elution confirm these results, as well as the presence of hexoses and of glucosamine in the albumin fraction of I.T.P. serum. No carbohydrates are found in the albumin fraction of normal serum.

2. The eluted albumin fraction of normal serum migrates as a uniform component in phthalate buffer at pH 4.6; under similar conditions, the albumin fraction from serum of patients with I.T.P. divides into two components. The faster component represents up to 80 per cent of the entire albumin fraction and contains all protein-bound hexoses and the greatest amount of glucosamine found in the albumin fraction.

3. These findings may be related to the presence in patients with I.T.P. of an autoimmune state against platelets.

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ANTIHEMOPHILIC GLOBULIN ASSAY FOLLOWING PLASMA INFUSIONS IN HEMOPHILIA

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INTRODUCTION

ANTIHEMOPHILIC globulin (A.H.G.) is a plasma clotting factor which is found in association with the fibrinogen fraction of normal blood, but is lacking in the blood of hemophilic patients. In the practical management of hemorrhage in the hemophiliac it is of value to have an appreciation of the result of plasma infusions on the A.H.G. level of the patient's blood and of the duration of this effect. It is also of importance to have a correlation between the A.H.G. level and the hemostatic effect. It was the object of this investigation to study these aspects in patients with hemophilia following the administration of plasma.

The therapeutic materials which are at present generally available for the management of the hemostatic defect in hemophilia are whole blood, fresh-frozen plasma, and the lyophil-dried plasma fraction I (Cohn). As a source of supply of A.H.G., whole blood is unsatisfactory. The blood must be fresh, because assay of the A.H.G. concentration of stored blood reveals that often there are only small amounts left after 3 to 4 days.[1] Furthermore, hemophiliacs are frequently the recipients of multiple transfusions and there is the danger of hemolytic transfusion reactions unless sensitive compatibility tests are employed; this may be impossible in an emergency. Fresh-frozen plasma is readily prepared; blood is collected as for transfusion purposes, the red cells separated, and the plasma kept at –20° C. Under these conditions of storage the A.H.G. survival is considerably better than in bank blood. The lyophil-dried A.H.G. fraction is relatively concentrated, but is expensive to prepare in comparison with the cost of fresh-frozen plasma. A theoretical disadvantage of the fresh-frozen plasma is the large volume required, with, in consequence, the possibility of circulatory overloading. In practice this problem has not been encountered, and, in this study, the A.H.G. was administered as plasma, generally fresh-frozen plasma.

The improvement in the clotting efficiency of hemophilic blood in consequence of therapeutic administration of plasma or plasma fractions can be

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assessed by a variety of "in vitro" techniques. These include the whole blood clotting time, the plasma recalcification time, the prothrombin consumption test,[2] the thrombin generation test,[3] the partial thromboplastin time test,[4] and the thromboplastin generation technique.[5] When used unmodified, the whole blood clotting time, the plasma recalcification time, and the prothrombin consumption test on whole blood are insensitive indices of clotting efficiency because small amounts of A.H.G. will produce disproportionately large correction. The plasma recalcification time and the prothrombin consumption technique can, however, be modified to produce a more sensitive assay. Other assay techniques which are available, are dependent on the partial thromboplastin time and on modifications of the thrombin generation test and the thromboplastin generation technique.

In this study use has been made of the whole blood coagulation time, the prothrombin consumption test, the thrombin generation technique, and the thromboplastin generation method. The last 3 have been modified to give assays and the thromboplastin generation technique in particular has been used extensively.

The assessment of the hemostatic level of A.H.G. is more difficult. An attempt has been made to correlate the level of antihemophilic globulin with the occurrence of hemorrhage following tooth extraction and the cessation of hematuria in hemophilic patients.

MATERIALS AND METHODS

Glassware.—All clotting times, of fibrinogen or of high-spun normal plasma, were carried out in tubes with an internal diameter of ½ in., maintained at 37° C.

Centrifugation.—This was done at 4° C. in a refrigerated centrifuge.

Reagents.—Calcium chloride (0.025 M), 3.8 per cent sodium citrate, and human fibrinogen were prepared as described by Biggs and Macfarlane.[6] The human brain thromboplastin was that described by Brown and Douglas.[7]

Preparation of Plasma.—The plasma was prepared by the Glasgow and West of Scotland Blood Transfusion Service. For each observation blood was collected from 4 donors. Generally donors of the same blood group as the recipient were used for the preparation of the fresh-frozen plasma, but donors of group A or AB were used for an emergency reserve. Donors with a high titer anti-B agglutinin or with anti-B hemolysin were excluded. The preservative-anticoagulant solution used was composed of 2 Gm. of disodium hydrogen citrate, 3 Gm. of dextrose, and sufficient water to make 100 ml. Four hundred and forty milliliters of blood was collected into the 100 ml. of preservative-anticoagulant solution.

Only donors giving blood at the Regional Transfusion Center were used so that the plasma could be separated immediately and frozen. Centrifugation of the blood was carried out at 1,800 r.p.m. for 30 minutes. The plasma was separated with aseptic and antiseptic precautions and stored frozen at −20° C.

The volume of plasma obtained for each infusion as a consequence of separation from 4 pints of blood varied from 860 ml. to 1,050 ml. The mean value was 960 ml.

In one observation the plasma infused had not been frozen; it was collected as described above and administered as soon as collected.

Administration of Plasma.—The plasma was thawed immediately before use by standing the bottle in a basin of warm water and was given to the hemophiliac by moderately rapid intravenous transfusion, the time of administration varying from 45 to 60 minutes.

The plasma was administered to hemophilic patients generally for therapeutic purposes, e.g., for tooth extraction, for hematuria, or hematoma formation. In one observation there was no known site of hemorrhage.

Collection of Specimens.—Blood was collected by clean venepuncture from the patients before the administration of the plasma, immediately after, and at 6, 12, 24, 48, and 72 hours from the time of starting the infusion. Standard sized 20 ml. syringes and wide bore needles (S.W.G. 18) were used. In order to avoid frothing of the blood the syringes were allowed to fill without traction being applied to the plunger. Before delivery of the blood to the tubes the needle was removed. Using a graduated centrifuge tube, 9 ml. of blood was mixed with 1 ml. of 3.8 per cent sodium citrate. These specimens were used for the assay of antihemophilic globulin by the thromboplastin generation test and the prothrombin consumption technique. The specimens were immediately centrifuged at 20,000 r.p.m. for 5 minutes in a high-speed centrifuge attachment and the plasma separated and stored at −20° C. until all the specimens had been collected. The antihemophilic globulin content was then assayed in these specimens in one experiment. In a small number of the observations an assay was also made on these specimens within half an hour of collection. The results of the assays on the fresh specimens and those frozen at −20° C. showed reasonable agreement.

The whole blood clotting time in 3 of the observations and the prothrombin consumption index in 2 were estimated on specimens collected simultaneously with those used for the assay of antihemophilic globulin. On 6 occasions specimens of plasma were collected for study on the thrombin generation technique. The plasma was collected as described above for use in the thromboplastin generation test except that the plasma separated was platelet-rich as a consequence of centrifugation at 500 r.p.m. for 30 minutes.

Whole Blood Coagulation Time.—This was carried out at room temperature in tubes with an internal diameter of 10 to 11 mm., maintained at ward temperature. One milliliter of blood was delivered to each of 2 tubes and these were gently inclined once every 5 minutes until coagulation occurred. The range of normal by this method was 10 to 25 minutes.

Thromboplastin Generation Technique of A.H.G. Assay.—The 30 observations described in this paper were carried out during a 4-year period of study of the problem. The method of assay using the thromboplastin generation technique was modified after the first 12 observations in which the method (A) described below was used. The assay was made by a second method (B) in the subsequent 18 infusions.

A. As described by Douglas.[8] The variable component in the system was the adsorbed plasma: before, immediately after infusion, and at 6, 12, 24, 48, and 72 hours later. These results were then assayed by using mixtures of a random normal adsorbed plasma in the specimen of adsorbed hemophilic plasma collected before the infusion.

B. In this modification the clotting time was estimated only at the 6-minute point after recalcification. Seven incubation mixtures were set up (e.g., before, after, 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours, or 100 per cent, 50 per cent, 25 per cent, 12 per cent, 6 per cent, 3 per cent, 0 per cent) and recalcified at 1-minute intervals. As soon as the seventh tube was recalcified the first was ready for test and the others became ready at 1-minute intervals thereafter in succession. The control again was a random normal subject and from the figures obtained on the mixtures of control in the hemophiliac before transfusion, a graph was prepared (e.g., as shown in Fig. 1).

It has been shown that the A.H.G. content of the blood of hemophiliacs is variable in accordance with the degree of severity of the condition.[9, 10] The severely affected hemophiliac with a greatly prolonged whole blood coagulation time has no A.H.G. The mild or moderately affected patient, with a normal or only slightly prolonged whole blood clotting time, may have up to 30 per cent A.H.G. The great majority of the infusions were given to severely affected hemophiliacs with negligible amounts of A.H.G.; 4 of the observations, however, were made on short clotting time hemophiliacs. Since the dilution

curve of the normal was made into the specimen of patient's plasma collected before starting the infusion the results in these particular observations represent the increment over the patient's initial level of A.H.G.

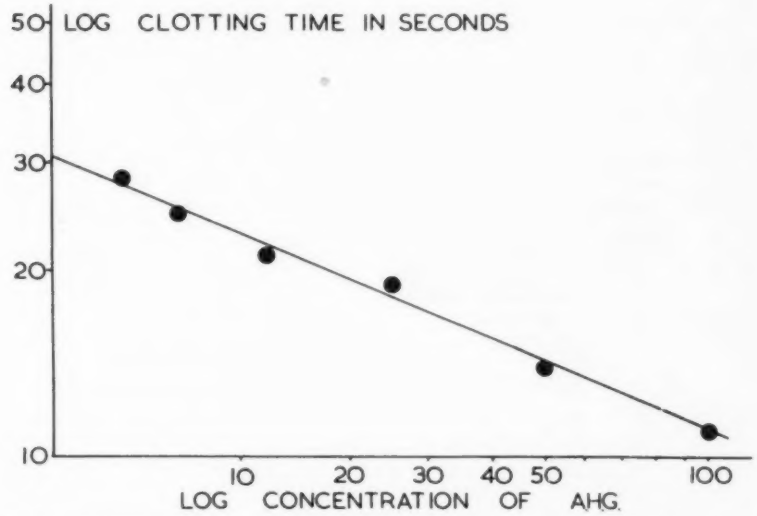

Fig. 1.—Thromboplastin generation technique (Method B) of A.H.G. assay. Example of a graph for the reading of results.

Prothrombin Consumption.—A. On whole blood as described by Merskey.[2]

B. On recalcified hemophilic platelet-rich plasma as described by Douglas and Cook.[11] The supply of low-spun hemophilic plasma was prepared from the patients before the administration of the infusion.

Thrombin Generation Technique.—In this method 0.5 ml. of low-spun plasma with 0.5 ml. of saline was recalcified with 0.5 ml. of 0.025 M calcium chloride and at 1-minute intervals 0.1 ml. transferred to 0.2 ml. of fibrinogen and the clotting times recorded. Any clot formed was removed on a wooden applicator stick. Mixtures were made of a normal plasma in the specimen of hemophilic plasma collected before starting the infusion. The test was carried out on each of the mixtures of normal in hemophilic plasma and on the specimens before and at intervals after the infusion. By comparing the readings at one specified minute interval (e.g., sixth minute) after recalcification, an assay was made on the infusion specimens.

RESULTS

Whole Blood Coagulation Times.—The results in 3 of the observations, respectively, were as follows: before, over 120 minutes, over 120 minutes, and over 120 minutes; after, 11 minutes, 11 minutes, and 14 minutes; 6 hours, 13 minutes, 14 minutes, and 12 minutes; 12 hours, 21 minutes, 25 minutes, and 30 minutes; 24 hours, 28 minutes, 30 minutes, and 60 minutes; 48 hours, 90 minutes, 70 minutes, and over 120 minutes; 72 hours, over 120 minutes, over 120 minutes, and over 120 minutes.

There was a correction to normal immediately after the infusion. This marked correction of the whole blood clotting time is to be compared with the

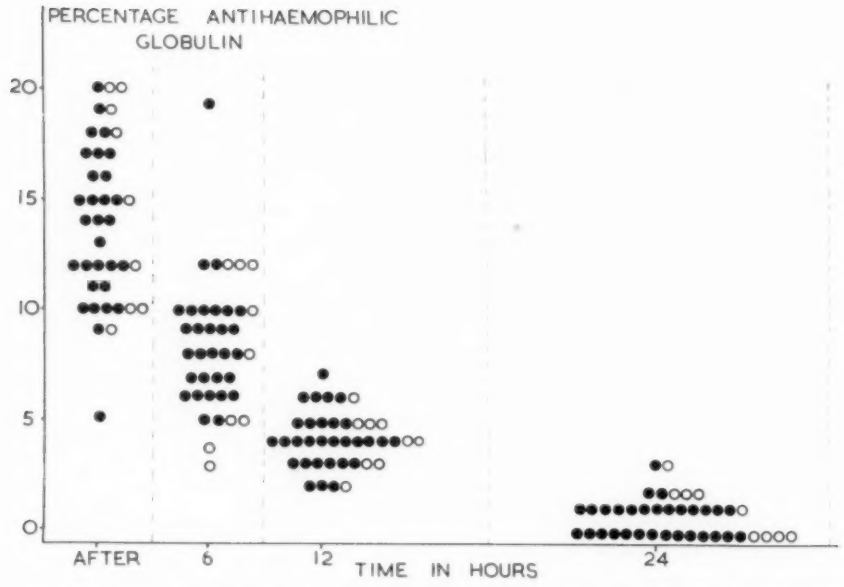

Fig. 2.—In this figure the results of the A.H.G. assays using the thromboplastin generation technique are shown as solid dots, for the specimens collected immediately after infusion, 6 hours, 12 hours, and 24 hours later. At 48 hours after infusion in 9 of the observations there were trace amounts of A.H.G. present and in the remainder no A.H.G. was demonstrable. There was no A.H.G at 72 hours in any of the observations.

To the right of the solid dots, recording the 30 assays on the thromboplastin generation technique, are circles representing the results of the 9 observations using the prothrombin consumption technique. In these 9 observations there was no demonstrable A.H.G. in the 48-hour and 72-hour specimens.

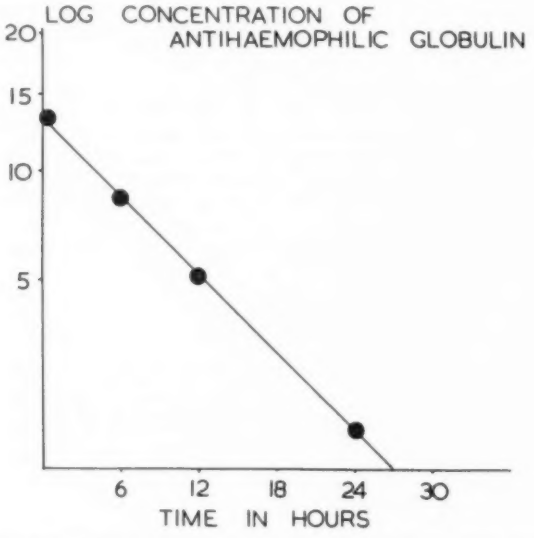

Fig. 3.—Antihemophilic globulin survival "in vivo." It will be observed that the half-life is approximately 9 hours.

very defective correction of thromboplastin generation as demonstrated below in the A.H.G. assay.

Thromboplastin Generation Technique of A.H.G. Assay.—Examples of methods (A) and (B) are shown in Table I and the results of the assays in Fig. 2.

TABLE I. EXAMPLES OF METHODS OF A.H.G. ASSAY

INCUBATION MIXTURE	THROMBOPLASTIN GENERATION TECHNIQUE: METHOD A (INCUBATION TIME IN MIN.)						METHOD B DUPLICATE READINGS (INCUBATION TIME IN MIN.)		PROTHROMBIN CONSUMPTION TECHNIQUE AT 45 MIN. (INCUBATION TIME IN MIN.)		THROMBIN GENERATION TECHNIQUE (INCUBATION TIME IN MIN.)
	1	2	3	4	5	6	6	6	¾	1½	6
Before	42	38	40	38	38	36	45	47	10	11	0
After	15	16	15	14	14	14	17	18	25	26	8.5
6 hr.	17	16	16	15	16	16	19	19	19	19	6.9
12 hr.	19	17	19	17	18	17	23	24	16	16	5.5
24 hr.	24	23	22	22	23	24	38	37	12	12	2.1
48 hr.	37	30	35	35	34	34	49	51	10	11	0
72 hr.	39	35	36	37	38	38	47	49	11	10	0
100 per cent	10	9	9	9	9	9	11	11	60	59	–
50 per cent	11	10	11	11	11	11	13	13	45	49	–
25 per cent	19	12	12	12	12	12	16	15	31	33	9.2
12 per cent	24	14	14	13	14	13	18	20	20	18	7.0
6 per cent	28	17	16	15	15	15	22	24	16	15	5.0
3 per cent	39	22	19	19	19	19	29	32	13	14	4.8
0 per cent	40	36	36	35	35	36	48	45	10	11	0
	Clotting times in seconds										Thrombin units
	Readings from these figures in percentages of A.H.G.										
After	12						18		19		21
6 hr.	6						12		12		12
12 hr.	3						5		5		7
24 hr.	1						1		1		5
48 hr.	0						0		0		0
72 hr.	0						0		0		0

The mean values of these 30 observations were then determined and in Fig. 3 the logarithm of the concentration of A.H.G. has been plotted against the time in hours. From this it can be seen that the half-life of A.H.G. is approximately 9 hours.

The mean rise in A.H.G. concentration as a consequence of the infusion of a liter of plasma to an adult hemophiliac was 13.5 per cent. By 6 hours this had fallen to 8.5 per cent, by 12 hours to 4.1 per cent, and by 24 hours to less than 1 per cent.

In all the observations the plasma used was fresh frozen except in one when the plasma was administered immediately after collection. In another of the observations there was no known site of hemorrhage at the time of the infusion. These observations were made in view of the possibility that the rapid rate of utilization of A.H.G. was related either to the method of storage of the plasma by freezing or to the consumption of A.H.G. for hemostatic purposes at a site of hemorrhage. The results on these two particular infusions were not dissimilar to the other observations and are included in Fig. 2.

Prothrombin Consumption Technique.—On whole blood: the results on two observations recorded as prothrombin consumption indices were, respectively, as follows: before, 146 per cent and 188 per cent; after, 27 per cent and 35 per cent; 6 hours, 33 per cent and 33 per cent; 12 hours, 85 per cent and 40 per cent; 24 hours, 100 per cent and 55 per cent; 48 hours, 190 per cent and 93 per cent; 72 hours, 220 per cent and 140 per cent. The levels of A.H.G. attained as a consequence of the infusion have restored the prothrombin consumption to within the normal range in the readings immediately after and at 6 hours. At 24 hours the trace of A.H.G. remaining was still sufficient to produce some correction of the prothrombin consumption, though by then outside the normal range. The restoration to normal prothrombin consumption is in contrast to the level of A.H.G. attained by the assay on the thromboplastin generation technique.

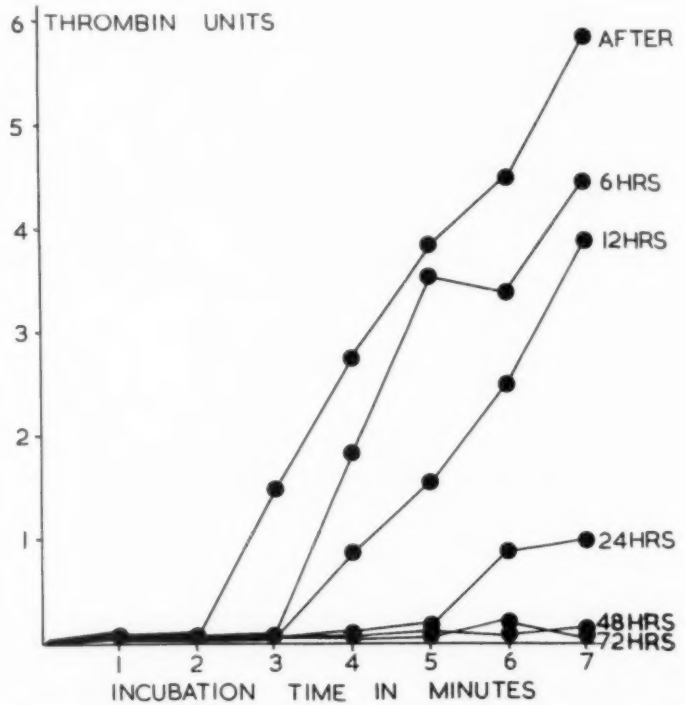

Fig. 4.—Thrombin generation technique. Thrombin development from plasmas collected at intervals as indicated on the diagram.

On recalcified hemophilic platelet-rich plasma: in Table I an example of this form of assay is shown and in Fig. 2 the results on 9 of the observations assayed by this technique.

Thrombin Generation Technique.—In 6 of the observations this technique was applied, and the mean of the results is shown in Fig. 4. In 2 of these,

dilution curves were made and the assay read over 3-minute intervals. An example of the method of assay is shown in Table I and the mean of the 6 figures obtained by this method were: after, 17 per cent; 6 hours, 11 per cent; 12 hours, 7 per cent; 24 hours, 1 per cent; 48 hours, nil; 72 hours, nil.

Estimation of the Hemostatic Level of Antihemophilic Globulin.—In 23 of the observations tooth extraction was carried out within one hour of completion of the infusion. During the 24 hours following the extraction there was bleeding in only one of the extractions. In four of the dental operations some bleeding started 24 to 48 hours after the completion of the infusion. In 15 of the extraction operations there was some hemorrhage between 48 and 72 hours. It is probable from this that the level of A.H.G. attained (13 to 14 per cent) in consequence of the infusions, had some hemostatic value.

In one observation the patient had hematuria for one week before the plasma was administered. Prior to starting the plasma infusion he passed a specimen of urine showing gross hematuria and was given 2 pints of water to drink to produce a diuresis. At 15-minute intervals from the time of starting the infusion further specimens of urine were collected. The 15- and 30-minute specimens still contained blood; the 45-minute specimen was obviously clearer, and the 60-minute specimen contained no obvious blood. To obtain hemostasis in this patient for this particular purpose an A.H.G. level of 12 per cent was effective.

DISCUSSION

This investigation confirms the difficulties involved in the therapeutic replacement of A.H.G. in the hemophiliac. In order to obtain a level of 14 per cent of A.H.G. in a hemophiliac, approximately a liter of plasma has to be given with a rapid infusion and this administered A.H.G. has been utilized in 24 hours. The maintenance of a level of 14 per cent A.H.G. would require the administration of a liter of plasma every 6 to 12 hours. This rapid utilization of A.H.G. is related neither to the method of storage of the plasma by freezing nor to its consumption for hemostatic purposes at a site of hemorrhage. The assays indicate that A.H.G. has a half-life of approximately 9 hours.

There is suggestive evidence from this study that a level of 14 per cent A.H.G. had some hemostatic value in control of bleeding from the sites described. The production, if possible, of higher levels of A.H.G. activity in the hemophiliac's blood is clearly desirable, and this has been described using preparations of A.H.G. from animal sources.[12-15] Although the levels of A.H.G. attained in this study were probably of some value in hematuria and in bleeding from tooth extraction, these are relatively minor tests of hemostatic efficiency. It is probable that for surgical procedures, involving trauma to larger vessels, a higher level of A.H.G. is required.

Preparations of Cohn's plasma fraction I are available as a source of A.H.G. for the treatment of hemophilia. The difficulty remains, however, of the amount required. Even if there is full recovery of the A.H.G. from the

plasma it would still require the fraction derived from a liter of plasma for administration every 6 to 12 hours to maintain even the low levels of A.H.G. attained with the infusions described in this investigation.

Until such time as concentrated preparations of A.H.G. from human plasma,[16] from animal plasma,[12-15] or from tissue[17] become available, whole human plasma must remain as the mainstay of therapy. As mentioned above, A.H.G. assays on the plasma of blood stored for transfusion purposes at 4° C. reveal that commonly there are only trace amounts left after 3 to 4 days. In plasma maintained at –20° C. from the time of collection, the preservation of A.H.G. is considerably better. Such plasma can be kept available by transfusion services to meet the emergency of bleeding in hemophilia.

The level of A.H.G. attained as a consequence of the infusions was found to vary from 5 per cent to 20 per cent. Pitney[9] observed that the level of A.H.G. in normal subjects varied from 65 per cent to 160 per cent. Others have found similar figures.[9, 10, 18] It is likely that the wide scatter in the results is in part due to this variation in the normal. This would influence the result in that a random normal plasma used as the control in each assay and to a lesser extent the content of the A.H.G. in the donor plasma would be variable.

Biggs[10] has found, after infusion of 1,000 ml. of fresh plasma, levels of A.H.G. between 18 per cent and 30 per cent. The figures in this investigation are slightly lower (5 to 20 per cent with a mean of 14 per cent). The volumes used here were often slightly less than 1,000 ml. and the plasma was prepared from blood collected in a relatively large volume of anticoagulant fluid (440 ml. of blood to 100 ml. of acid citrate dextrose solution). The plasma used was maintained frozen from the time of collection for a period varying from a few days to several weeks. Though the frozen samples often maintain activity well, there may have been some over-all deterioration of A.H.G. activity as compared with the use of fresh plasma as described by Biggs.[10]

These observations confirm previous reports that the correction of the whole blood clotting time does not mean restoration to a normal coagulation mechanism. The whole blood clotting time and the prothrombin consumption were reduced to normal when the A.H.G. assay revealed only 15 to 20 per cent A.H.G. to be present.

Whether the rapid disappearance of antihemophilic globulin in the hemophiliac represents the physiologic utilization of the factor is problematical. Langdell and associates[19] administered plasma to hemophilic and normal dogs. The utilization in the normal dogs was as rapid as in the hemophilic dogs.

SUMMARY

Antihemophilic globulin (A.H.G.) has been assayed in hemophiliacs following the administration of 1 liter of plasma. In 30 observations, using the thromboplastin generation technique as a method of A.H.G. assay, a mean level of 14 per cent A.H.G. was attained on completion of the infusion. The half-life of the administered A.H.G. was approximately 9 hours and less than 1 per cent was demonstrable at 24 hours. Similar levels of A.H.G. were found using a

thrombin generation method and a prothrombin consumption technique on recalcified hemophilic plasma. The observations suggested that the levels of A.H.G. attained by these infusions probably provided some hemostatic effect in hematuria and tooth extraction in hemophilic patients.

I wish to thank Professor L. J. Davis for his interest in this investigation and guidance in the preparation of this manuscript. I am also grateful for the helpful cooperation of Dr. J. Wallace, Director of the Glasgow and West of Scotland Blood Transfusion Service, and for the valuable technical assistance of Miss Kathleen Mair.

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SERUM VITAMIN B_{12} CONCENTRATIONS DETERMINED BY L. LEICHMANNII ASSAY IN PATIENTS WITH NEOPLASTIC DISEASE

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THIS paper presents details of a modification of the *U. S. Pharmacopeia XV Lactobacillus leichmannii* method[1] of assay developed specifically for determinations of vitamin B_{12} in serum, and reports the results of a survey of serum B_{12} levels measured by this technique in 31 normal controls and 93 patients with neoplastic disease and certain other conditions.

METHODS

The U.S.P. method was followed closely with the exceptions detailed below.

Assay Preparation.—All serum samples were drawn from fasting subjects. To each milliliter of serum, 2 ml. of an aqueous extraction solution was added. Each 100 ml. of extraction solution, prepared just prior to use, contained 1.29 Gm. of disodium phosphate, 1.1 Gm. of anhydrous citric acid, and 0.1 Gm. of sodium metabisulfite. Sodium metabisulfite concentrations of less than 0.001 Gm. per milliliter resulted in incomplete extraction of B_{12} from serum, while greater concentrations (assuming 0.5 ml. of extraction solution present in each final assay tube) resulted in inhibition of growth of the organism. The pH of this extraction solution was approximately 4.6.

The mixture of serum and extraction solution was autoclaved at 121° C. for 10 minutes, centrifuged, and duplicate 0.75 ml. and 0.3 ml. portions of the supernatant (containing 0.25 and 0.1 ml. of serum, respectively) were transferred to the final assay tubes. Sufficient extraction solution was added where necessary to bring its volume in each final assay tube to 0.5 ml. The final volume of each tube (before addition of assay medium) was brought to 5.0 ml. with distilled water.

Assay Medium.—Five milliliters of Bacto B_{12} Assay Medium,† U.S.P., containing 85 Gm. of solid material per liter of distilled water, was added to each final assay tube.

Standard Curve.—A standard B_{12} solution containing 0.050 mμg per milliliter was made up from a B_{12} stock solution prepared monthly from crystalline B_{12} according to U.S.P. instructions. With each assay, 9 dilutions of the working standard, each in duplicate, representing from 0.025 to 0.225 mμg of B_{12} were added to final assay tubes to comprise the standard curve.

Inoculum.—The organism, *Lactobacillus leichmannii*,‡ was transferred daily in Bacto B_{12} Inoculum Broth,† U.S.P. A 20-hour growth was centrifuged and washed 3 times in saline. One drop of material, diluted to give a reading of about 72 per cent transmittance at 600 mμ, in the Coleman Universal spectrophotometer (corresponding to approximately 0.5 mg. cells per 10 ml. of saline), was inoculated into each tube.

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‡American Type Culture Collection No. 7830.

Procedure.—After either standard B_{12} solution or supernatant from the serum extraction was introduced into the assay tubes, the complementary extraction solution (if required), distilled water, and assay medium were added. The tubes were placed in racks covered with gauze-lined aluminum trays and autoclaved at 121° C. for 5 minutes. They were cooled and, after inoculation as outlined above, were incubated at 37° C. for 20 hours. The per cent transmittance of each tube was then determined in a Coleman Universal spectrophotometer against an uninoculated reagent blank at a wave length between 610 and 623 mμ. The precise wave length for a given assay was ascertained by determining the maximum per cent transmittance of the uninoculated blank for that assay. The per cent transmittance of each tube containing serum extract was converted to B_{12} concentration by reference to the standard curve run with each assay.

TABLE I. RECOVERY EXPERIMENTS

SERUM B_{12} CONCENTRATION MμG B_{12}/ML. OF SERUM	MμG B_{12} ADDED	MμG B_{12} RECOVERED	PER CENT RECOVERY
0.550	0.050	0.041	82
0.550	0.100	0.100	100
0.550	0.025	0.030	120
0.550	0.050	0.051	102
0.380	0.100	0.087	87
0.380	0.050	0.049	98
0.520	0.100	0.090	90
0.520	0.050	0.055	110
0.450	0.100	0.107	107
0.450	0.050	0.045	90
0.530	0.100	0.093	93
0.530	0.050	0.052	104
			Mean = 98.6%
			S.D. = 10.83%
			$S_{\bar{x}}$ = 3.13%

RESULTS

The recoveries from 5 different sera containing various amounts of added crystalline B_{12} are recorded in Table I.

Reproducibility at various B_{12} levels was tested by running the standard curve with 16* replicates at each of 9 B_{12} concentrations. As shown in Table II, the deviations about the mean per cent transmittance at each B_{12} concentration ranged from 2.7 to 10 per cent of the mean. As a further check on reproducibility, 16* replicate assays were performed on each of 9 different quantities of serum from a patient with an elevated B_{12} concentration. The deviations about the mean per cent transmittance for each quantity of serum ranged from 3.4 to 10.5 per cent of the mean. The deviations about the mean quantity of B_{12} in each tube ranged from 5.4 to 22.2 per cent of this mean (See Table II). The per cent values are the quotient times 100 of the standard deviation divided by the mean.

DISCUSSION

The *Euglena gracilis* method of Ross[2] has been used widely from the determination of serum vitamin B_{12} concentrations. It has the advantages of

*See Table II for exceptions.

being sensitive and specific.[3] It has the disadvantage of requiring 8 to 10 days for incubation of the organism. In addition, although Ross has reported "usually almost complete" recoveries, Pitney and associates[4] have found recoveries in the range of 50 per cent. Harris[5] has reported recoveries of 70 to 90 per cent, although his method since has been modified.[6]

TABLE II. REPRODUCIBILITY EXPERIMENTS

PER CENT TRANSMITTANCE			B_{12} MμG PER TUBE	$S_{\bar{x}}$ OF B_{12} VALUES OBTAINED	ML. OF SERUM PER TUBE
$\bar{x}$	S. D.	$S_{\bar{x}}$			
A. *Standard Curve*					
83.1	2.22	0.79*	0.000		
61.1	2.20	0.56	0.025		
46.9	2.69	0.67	0.050		
34.5	2.22	0.56	0.075		
25.5	1.47	0.37	0.100		
20.5	1.39	0.35	0.125		
17.1	1.36	0.34	0.150		
14.4	1.44	0.36	0.175		
12.9	0.98	0.25	0.200		
10.8	1.03	0.28†	0.225		
B. *Serum*					
81.9	4.03	1.43*	0.002		0
66.7	2.24	0.56	0.018	0.001	0.0016
55.3	1.97	0.49	0.035	0.001	0.0032
44.1	2.82	0.71	0.054	0.001	0.0048
35.3	2.06	0.52	0.073	0.001	0.0064
29.0	2.84	0.73‡	0.092	0.002	0.0080
23.8	2.32	0.58	0.110	0.002	0.0096
20.2	1.78	0.45	0.127	0.003	0.0112
16.8	1.76	0.44	0.152	0.003	0.0128
15.4	1.18	0.30	0.165	0.003	0.0144

*n = 8.
†n = 14.
‡n = 15.

A. Reproducibility tested by running 16 replicate determinations at each of 9 B_{12} concentrations on the standard curve. The mean per cent transmittance is listed in column 1, the standard deviation in column 2, the standard error of the mean in column 3, and the quantity of B_{12} in each tube in column 4.

B. Reproducibility tested by running 16 replicate assays on each of 9 different quantities of serum from a patient with an elevated B_{12} concentration. Columns 1 to 4, as above. The standard error of the mean B_{12} value for each tube is listed in column 5 and the milliliter of serum per tube in column 6.

The principal advantage of the *L. leichmannii* method is the 20-hour incubation time. The method is somewhat less specific than the *E. gracilis* technique. *L. leichmannii* will respond with growth to the presence of desoxyriboses if purine bases are also present in the medium. However, 1,000 times as much desoxyribose as B_{12} is needed for growth.[7] Hence, this factor is largely eliminated from serum by the dilution involved in the method. Although desoxyribose concentrations in serum have not been measured, the B_{12} recovery data presented here together with that of Rosenthal and Sarett[8] indicate that the *L. leichmannii* assay is sufficiently specific and quantitatively accurate for satisfactory determinations. Its convenience and rapidity compared to the *E. gracilis* assay suggest it as the method of choice in assaying B_{12} concentrations in serum.

RESULTS OF SURVEY

Vitamin B_{12} concentrations were measured in the sera of 31 normal individuals. The data are presented in Table III.

TABLE III. LEVELS IN 31 NORMAL SUBJECTS

MEAN (MμG/ML.)	RANGE (MμG/ML.)	S.D. (MμG/ML.)	$S_{\bar{x}}$ (MμG/ML.)
0.533	0.260-0.850	0.166	0.030

Vitamin B_{12} concentrations were measured by 109 determinations in the sera of 93 patients. Elevated levels were defined as those 2 standard deviations or more above the normal mean (i.e., greater than 0.865 mμg/ml.). Of these 109 determinations, 35 were elevated. These 35 elevated levels were found in 23 patients with 12 different diseases. Twenty-four other patients (with 8 of these same 12 diseases) had normal B_{12} levels. In Table IV, the number of elevated and normal B_{12} levels for each disease entity characterized by an elevated serum B_{12} concentration is presented.

TABLE IV. DATA ON PATIENTS WHOSE DISEASES DEMONSTRATED ELEVATED SERUM B_{12} CONCENTRATIONS

DISEASE	TOTAL PATIENTS	NUMBER OF DETERMINATIONS	NUMBER PATIENTS WITH ELEVATED LEVELS	RANGE OF ELEVATED LEVELS (MμG/ML.)	NUMBER PATIENTS WITH NORMAL LEVELS
Chronic myelocytic leukemia	5	11	4	1.2 -12.0	1*
Acute myelocytic leukemia	9	9	6	1.4 - 3.1	3†
Chronic lymphatic leukemia	3	4	1‡	0.92- 1.6	2
Malignant melanoma	8	10	3	1.55- 8.67	5
Carcinoma of cervix	4	4	2	1.1 - 1.57	2
Carcinoma of sigmoid colon	3	3	1	3.10	2
Carcinoma of breast	8	9	1	4.10	7
Hodgkin's disease	3	3	1§	1.90	2
Adenocarcinoma, primary (?)	1	1	1	1.67	
Squamous carcinoma, primary (?)	1	1	1	1.02	
Cirrhosis	1	1	1	1.1	
Myeloid metaplasia	1	5	1	1.07- 2.4	

*In aleukemia phase of disease.
†In 2 of these 3, the marrow was read as acute monocytic or myelocytic leukemia; in the third, as Pelgeroid type of acute myelocytic leukemia.
‡Patient also has nephrotic syndrome.
§Had received "B" vitamin injections.

Of the 93 patients, 9 had serum B_{12} concentrations 2 or more standard deviations below the normal mean (i.e., less than 0.201 mμg/ml.). Of this group, 2 were strict vegetarians and 1 each had mycosis fungoides, malignant carcinoid, and carcinoma of the kidney, lung, mouth, bladder, and tongue. The serum B_{12} concentrations ranged between 0.075 and 0.175 mμg/ml.

TABLE V. DATA OF 12 PATIENTS WITHOUT LEUKEMIA BUT WITH ELEVATED B_{12} CONCENTRATIONS

DISEASE	WHITE BLOOD COUNT	BSP RETENTION (% AT 45 MIN.)	CEPH. CHOL. FLOC.	THYMOL. TURB.	ALKALINE PHOSPHATASE (BESSEY-LOWRY U.)	TOTAL BILIRUBIN (MG. %)	POST-MORTEM FINDINGS
Melanoma	55,000	22	0	4	1.8	0.4	Widespread metastases; diffuse liver metastases
Melanoma	4,100	1	2+	2	3.5	0.4	Widespread metastases; most of liver replaced by tumor
Melanoma	6,200	14	0	1	12.8	0.3	Widespread metastases; extensive large hepatic metastases
Carcinoma of cervix	15,200	4	0	3	1.9	0.4	One small tumor nodule in liver
Carcinoma of cervix	6,700		0	1	1.7	0.3	Still living
Carcinoma of colon	8,000	19	0	3	2.0	0.2	Still living; evidence of hepatic metastases on I^{131} Rose Bengal scanning
Carcinoma of breast	7,500		0	8	10.4	4.2	Extensive hepatic and bone metastases
Hodgkin's disease	14,900	35	0	1	10.0	0.7	Still living
Adenocarcinoma, primary (?)	7,400	10	0	11	3.2	0.3	Still living
Undifferentiated carcinoma, primary (?)	7,200	20	1+	2	4.8		Widespread metastases; extensive hepatic metastases
Myeloid metaplasia	212,000	8	1+	8	7.4	9.5	Hepatosplenomegaly extramedullary hematopoiesis in liver, extensive
Cirrhosis	5,000		4+	4	6.2	7.6	Extensive portal cirrhosis

Thirty-seven patients had serum B_{12} concentrations within 2 standard deviations of the normal mean. These included patients with acute lymphatic leukemia, 5; granuloma fungoides, 4; carcinoma of the mouth and of the testicle, 3 each; carcinoma of the bladder and of the esophagus, 2 each; carcinoma of the prostate, maxillary antrum, thyroid, stomach, endometrium and vulva, 1 each; and malignant carcinoid, lymphosarcoma, chondrosarcoma, multiple myeloma, leukemoid reaction, eosinophilic granuloma of bone, psoriasis with rheumatoid arthritis, myxedema, diabetes with tuberculosis, anemia possibly due to chronic myelocytic leukemia, ileal resection, and neuropathy of unknown etiology, 1 each.

Elevated serum B_{12} concentrations have been reported in patients with myelocytic leukemia,[9] polycythemia vera, carcinomatosis with leukemoid reactions and myelosclerosis.[10] Among the leukemic patients in this study, 4 of 5 with chronic myelocytic leukemia and 6 out of 9 with acute myelocytic leukemia were found to have elevated serum B_{12} levels. Only 1 of 3 patients with chronic lymphocytic leukemia and none of 5 with acute lymphocytic leukemia had elevated levels. The patient with chronic myelocytic leukemia whose level was normal was in the aleukemic phase of her disease. The patient with chronic lymphocytic leukemia whose level was elevated had the nephrotic syndrome apparently antedating his leukemia.

Of the 12 nonleukemic patients with elevated serum B_{12} concentrations, 10 had at least 1 abnormal liver function test. Autopsies on 8 of these patients revealed hepatic involvement, often extensive, in all. In Table V, the findings in these 12 patients are listed. The liver functions tabulated are those measured closest to the date on which blood for B_{12} determinations was drawn.

These findings suggest a relation between hepatic metastases and serum B_{12} elevations. They are in accord with the findings of such elevations in some patients with cirrhosis of the liver[6] and acute hepatitis.[11] Since not all patients with hepatic metastases have elevated serum B_{12} concentrations, if hepatic involvement is casually related to elevated serum B_{12} levels, the particular type of liver injury associated with the elevations remains to be discovered.

SUMMARY

1. A method for serum B_{12} concentration assay using *L. leichmannii* is described in detail. Recovery and reproducibility data are presented.

2. The serum B_{12} concentrations of 31 normals and 93 patients with neoplastic disease and other conditions are reported.

3. Most patients with myelocytic leukemia, acute and chronic, have elevated serum B_{12} levels. Only 1 of 3 patients with chronic lymphocytic leukemia had an elevated level.

4. Most patients with nonleukemic neoplastic disease have normal serum B_{12} concentrations. Those patients with elevated levels display a high incidence of abnormal liver function tests and of gross hepatic metastases.

We are indebted to Miss Helen Noble and Mrs. Virmetta Gilmore for their assistance in developing the assay method and conducting the survey.

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THE EFFECT OF DIET UPON ACUTE HYDRALAZINE TOXICITY IN WHITE RATS

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HYDRALAZINE, widely used in the treatment of hypertension, has also roused interest for an unrelated reason, i.e., its chronic administration has produced in a few patients and also in dogs a syndrome similar to or identical with systemic lupus erythematosus.[1] It is conceivable that investigation of the metabolic effects of hydralazine will provide clues to the pathogenesis or treatment of lupus. Therefore, it seemed worth while to test the possibility that gross differences in diet, known to affect profoundly the body's metabolism,[2] would alter the susceptibility of white rats to hydralazine poisoning.

METHOD

Young female albino rats (Wistar strain) were divided into the following dietary groups: (1) Control (stock diet), (2) high protein, (3) high carbohydrate, and (4) high fat. The diets (Table I) all contained adequate vitamins and minerals and have been found by Roberts and others to support normal growth and behavior.[2-5] In the first experiment 50 control animals were used; 25 were employed in each of the other 3 groups.

TABLE I. PERCENTAGE OF TOTAL CALORIES SUPPLIED BY PRINCIPAL NUTRIENTS IN DIETS (EXPERIMENT 1)

DIET	CARBOHYDRATE (PER CENT)	FAT (PER CENT)	PROTEIN (PER CENT)
Control	58	9	33
High protein	Initial, 20; then 31 to 25	Initial, 10; then 19 to 15	Initial, 70; then 50 to 60
High carbohydrate	83	2	15
High fat	0	85	15

In the second experiment the above groups were modified slightly to ensure identical protein content in the control, high-carbohydrate, and high-fat diets, and also to allow progressive adaptation of the high-protein animals to an increasingly higher protein intake (Table II). Thirty female Wistar rats were used in the high-protein and in the high-carbohydrate groups and 29 in each of the other two groups. The diets were composed of the following elements: dextrose for the carbohydrate, corn oil for the fat, and lactalbumin for the protein. In addition, for each 10,000 calories supplied by these basic foods the following substances were given: 45 Gm. of vitamin B mixture (Lederplex), 30 drops of oleum percomorphum, 300 Gm. of bulk (Alphacel), and 120 Gm. of Osborne-Mendel salt-mineral mixture "W."

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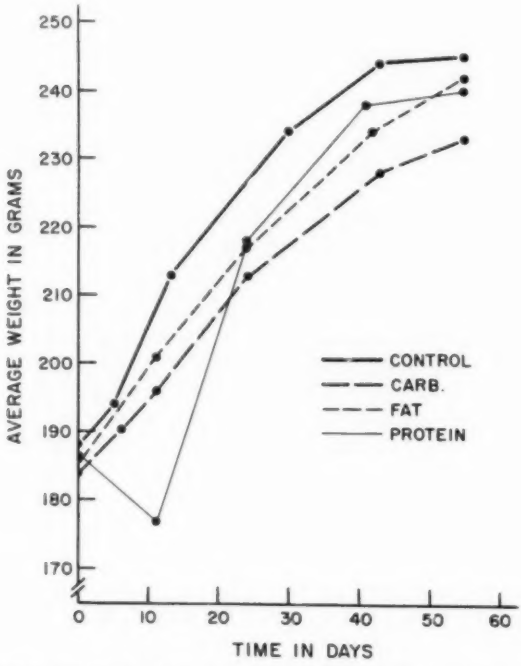

Fig. 1.—Average weight of white rats fed control and experimental diets (Experiment 1).

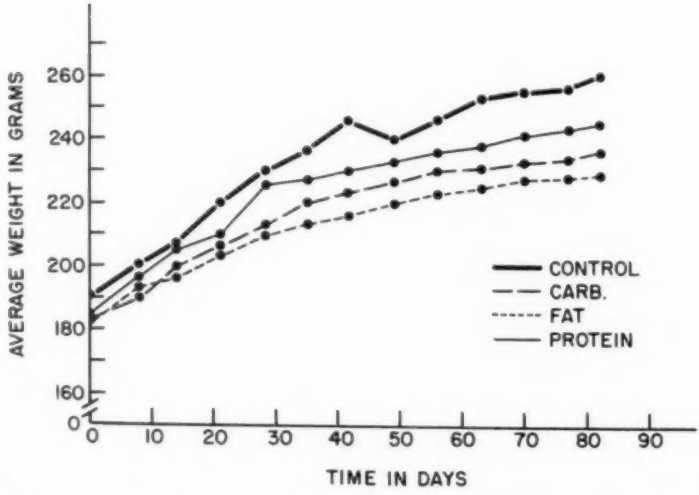

Fig. 2.—Average weight of white rats fed control and experimental diets (Experiment 2).

TABLE II. PERCENTAGE OF TOTAL CALORIES SUPPLIED BY PRINCIPAL NUTRIENTS IN DIETS (EXPERIMENT 2)

DIET	CARBOHYDRATE (PER CENT)	FAT (PER CENT)	PROTEIN (PER CENT)
Control	43	42	15
High protein	32 to 10	32 to 10	35 to 80
High carbohydrate	83	2	15
High fat	0	85	15

When the animals in the first experiment had been on their diets for 54 days and those in the second experiment for 82 days, all of the animals were given an aqueous hydralazine solution intraperitoneally, 40 mg. per kilogram. (This dose was found in preliminary experiments to be close to the LD_{50} for intraperitoneal administration, and others had found the intravenous LD_{50} in Wistar rats to be 34 mg. per kilogram.[6]

In the third experiment 29 rats were given hydralazine, 40 mg. per kilogram intraperitoneally, and 16 of them were given immediately thereafter approximately double an equimolar dose of pyridoxine hydrochloride by the same route. The remaining 13 animals were given normal saline intraperitoneally, the volume of saline equaling the volume of pyridoxine HCl solution that would have been given on the twice-equimolar basis.

In the fourth experiment 10 rats were fed the diets used in the second experiment. Four were given the control diet, and 2 were given each of the other 3 diets. After 22 days, status epilepticus was induced in two of the control rats by injecting 80 mg. per kilogram of hydralazine intraperitoneally, and then all 10 of the animals were killed with inhalation ether. The brains of these animals were removed immediately after death and emulsified separately in a Potter-Elvehjem tissue grinder. The homogenate was brought to a volume of 20 ml. with 75 per cent alcohol. The filtrate was removed by centrifugation and evaporated to a constant volume of 10 ml. A 2 ml. aliquot was diluted with an equal amount of 75 per cent alcohol. Aliquots containing 0.05 ml. were used for two-dimensional paper chromatography. Butyl alcohol and phenol were used as solvents, and ninhydrin for color development.[7, 8]

RESULTS

All of the animals in the first experiment gained weight satisfactorily and at approximately the same rate except for those eating the high-protein diet (Fig. 1). The initial high-protein diet caused bloody diarrhea and weight loss, but diarrhea promptly disappearad with a lower and gradually increased protein ration, and subsequent weights were close to normal (Table I and Fig. 1). In the second experiment the rats in Group 2 were fed a moderate protein diet at the beginning, and with gradual augmentation of the protein intake the growth of these animals kept pace with the growth of the others (Fig. 2). All animals in the second experiment appeared vigorous and healthy. It seemed that the high-carbohydrate animals were naturally more active than the others, but no accurate measurements of activity were made.

After injections of the hydralazine most of the animals first became hyperactive and bold, but after 5 to 20 minutes they were drowsy, inactive, and somewhat ataxic. After an hour or more some of the animals developed involuntary twitching and eventually violent generalized convulsions. Very few animals that once convulsed ultimately survived. Most of them perished within 2 to 24 hours, with agonal tetanic respiratory arrest. Survival in the first two experiments is summarized in Tables III and IV. No evidence was

obtained for an effect of the high-protein diet upon the mortality, but the high-carbohydrate diets increased the death rate, while the high-fat diets were protective.

TABLE III. EFFECT OF DIET UPON SURVIVAL OF WHITE RATS GIVEN 40 MG./KG. OF HYDRALAZINE INTRAPERITONEALLY

DIET	NO. ANIMALS	NO. SURVIVORS	χ^{2*}	P
Control	50	23	—	—
High protein	25	9	1.00	Approx. 0.30
High carbohydrate	25	1	17.7	< 0.01
High fat	25	17	4.8	Between 0.01 and 0.02

*Chi Square (χ^2) assumes no difference between control and experimental groups (Experiment 1).

TABLE IV. EFFECT OF DIET UPON SURVIVAL OF WHITE RATS GIVEN 40 MG./KG. OF HYDRALAZINE INTRAPERITONEALLY

DIET	NO. ANIMALS	SURVIVORS	χ^{2*}	P
Control	29	7	—	—
High protein	30	5	1.50	>0.2
High carbohydrate	30	2	5.68	<0.02
High fat	29	22	42.2	<0.01

*Chi Square (χ^2) assumes no difference between control and experimental groups (Experiment 2).

TABLE V. EFFECT OF INTRAPERITONEAL PYRIDOXINE UPON THE SURVIVAL OF WHITE RATS GIVEN 40 MG./KG. OF HYDRALAZINE (EXPERIMENT 3)

NO. OF RATS	DOSE OF PYRIDOXINE	NO. OF SURVIVORS	χ^2	P
16	80 mg./Kg.	7	0.16	Approx. 0.90
13	0	5	—	—

Pyridoxine did not protect the rats from hydralazine poisoning (Table V).

Chromatographic estimation of the amino acids in brain tissue was very similar in all animals tested. In the two control animals killed with inhalation ether, the following free amino acids (listed in order of relative concentration) were identified: glutamic acid, glutamine (a dipeptide), γ-aminobutyric acid, serine, glycine, aspartic acid, cystine (as cysteic acid), α-alanine, threonine, an unknown spot below serine and glycine, leucine, and lysine. No significant change was noticed in the animals killed after hydralazine injection. A few differences, of questionable significance, were noted in the other animals. By visual observation no difference in the concentration of the amino acid spots was noted between the control and high-fat animals. As compared with the controls, an increase in the serine spot and a decrease in the glycine spot were observed for the high-protein and high-carbohydrate groups.

DISCUSSION

Under the conditions of these experiments, a high-carbohydrate diet lowers and a high-fat diet raises the resistance of white rats to acute hydralazine intoxication. Whether the altered resistance is a nonspecific one, tending to potentiate or prevent convulsions, for example, or whether various diets specifically antagonize hydralazine poisoning to different degrees remains to be

seen. While a high-fat diet might inhibit convulsions, through ketosis, there is no reason to believe that a high-carbohydrate diet should do the converse. For short periods of time carbohydrate feeding promotes water retention and fats induce diuresis, changes that affect susceptibility to seizures, but water balance is restored in a matter of days.[9]

The convulsant property of thiosemicarbazide has been found to reside in its antagonism of pyridoxal phosphate, which is essential to the intracerebral transamination that produces γ-aminobutyric acid; the latter is probably an inhibitor of synaptic transmission in the brain.[10, 11]

The third experiment reported here suggests that pyridoxine antagonism is not essential to the convulsant property of hydralazine. The fourth experiment establishes the fact that under the conditions of these experiments hydralazine does not depress the cerebral manufacture of γ-aminobutyric acid. Pyridoxine deficiency is certainly produced by several hydrazides,[12] and these acute experiments do not imply that hydralazine poisoning is not accompanied by pryidoxine loss or inhibition. The experiments do show that hydralazine is capable of producing convulsions despite a plethora of pyridoxine and without depressing the cerebral concentration of γ-aminobutyric acid.

Hydrazides are hydrolyzed in vivo to form hydrazines, which are highly toxic convulsants.[13] One can only guess the manner in which diet affects the poisoning, nor can one by any means predict that the dietary effects observed in acute hydralazine intoxication would be seen with chronic administration of smaller doses. It might be profitable, however, to explore the problem further. The well-known liability of systemic lupus erythematosus to prolonged exacerbations[14] could be explained by a relative enzyme deficiency, one that happens also to be encouraged by hydralazine. If so, purposeful escape from the handicaps of a specific enzyme deficiency remains a possibility, and hydralazine poisoning might be one means of searching for the escape.

SUMMARY

A high-carbohydrate diet increased and a high-fat diet decreased the susceptibility of white rats to acute hydralazine poisoning. Pyridoxine administration was not protective, and neither the diets nor the drug itself changed the cerebral concentration of γ-aminobutyric acid.

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RELIABILITY OF ANTIBIOTIC SENSITIVITY TESTS AS DETERMINED BY A SURVEY STUDY

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AUTHORITIES in the field of infectious diseases agree that clinical diagnosis and identification of the causative organism are important criteria in the selection of the proper therapeutic agents for bacterial infections.[1] Opinions differ as to the relative importance of testing the antibiotic sensitivity of bacterial strains for this purpose. Investigators who have been critical of antibiotic sensitivity tests have stressed the difference between the in vitro conditions of the tests and the environment in which the antibiotic must act in vivo.[2, 3] Variations in the results of antibiotic sensitivity tests also are caused by differences in methodology and the general use of a bacteriostatic rather than a bactericidal end point.[4]

The routine use of antibiotic sensitivity tests has been especially criticized as unnecessary. The range of sensitivity for many of the bacterial genera to various antibiotics has been established and is within narrow limits. Also the pharmacologic nature of some antibiotics imposes narrow therapeutic limits. In many instances the clinical situation requires the initiation of treatment before laboratory results are available.

Despite these criticisms and inadequacies, routine antibiotic sensitivity testing has become a practice of many physicians. This increased load of tests has required quick and easy methodology. In the bacteriology laboratories of most hospitals commercial antibiotic disks are used. The results of these tests influence patient care and collectively they form a frame of reference by which physicians judge the applicability of antibiotics for clinical use. It seemed appropriate, therefore, to examine the validity of the tests for determining antibiotic sensitivity in routine use. In the present study the reliability of such tests was investigated by a survey of results obtained with standard bacterial strains in 9 major teaching hospitals in Chicago.

MATERIALS AND METHODS

Bacterial Strains.—Identical subcultures of 5 strains each of 5 different genera of bacteria were distributed to the clinical bacteriology laboratories of 9 Chicago hospitals for antibiotic sensitivity tests according to their routine procedure. Five to ten antibiotics were tested against each strain. The species of bacteria distributed were *Micrococcus pyogenes* var. *aureus* (coagulase-positive), *Esch. coli*, *Aerobacter aerogenes*, *Proteus mirabilis*,

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TABLE I. ANTIBIOTIC SENSITIVITY TEST METHODS IN CURRENT USE BY 9 CHICAGO HOSPITALS STUDIED IN THIS SURVEY

HOSPITAL	INOCULUM	TEST MEDIA	DISKS USED	INCUBATION PERIOD	METHOD OF READING END POINT	INTERPRETATION OF TEST RESULTS
1	Swab from 3 hr. growth in B.H.I. broth	B.H.I. Agar	Bacto*: 3 concentrations	Overnight (18 hr.)	Presence or absence of zone of inhibition	Zone of inhibition at low concentration = sensitive, at middle concentration = moderately sensitive, at high concentration = moderately resistant. No zone of inhibition = resistant
2	Loopful from colonies on original plate	Human Blood Agar	Bacto*: 1 concentration	Overnight (18 hr.)	Size of zone of inhibition	Large zone of inhibition = ++++ (sensitive), small zone of inhibition = +. No zone of inhibition = resistant
3	Swab from overnight growth in B.H.I. broth	Penassay Agar	Multidisks†: 2 concentrations	Overnight (18 hr.)	Presence or absence of zone of inhibition	Zone of inhibition at low concentration = sensitive, at high concentration = moderately resistant. No zone of inhibition = resistant
4	One loopful of 5 hr. B.H.I. broth growth to plate. Spread with glass rod	B.H.I. Agar	Bacto: 3 concentrations	Overnight (18 hr.)	Presence or absence of zone of inhibition	No interpretation. Reported as read, e.g., sensitive to 30 μg of antibiotic
5	Loopful from colonies on original plate	Trypticase Soy Blood Agar	Multidisks†: 2 concentrations	Overnight (18 hr.) sometimes 6 hr.	Presence or absence of zone of inhibition	No interpretation. Reported as read, e.g., sensitive or resistant to high concentration. Sensitive or resistant to low concentration
6	Swab from 1-3 hr. growth in B.H.I. broth	Human Blood Agar	Desi-Disk Rings‡: 2 concentrations	Overnight (18 hr.) sometimes 6 hr.	Size of zone of inhibition	Zone sizes related to nomogram standardized by tube dilution method. Reported as +, ++, +++, or resistant
7	Swab from 4-6 hr. growth in trypticase soy or B.H.I. broth	Heart Infusion Agar	B.B.L. Disks§: 2 concentrations	Overnight (18 hr.)	Presence or absence of zone of inhibition	Zone of inhibition with low concentration = sensitive, with high concentration = moderately sensitive. No zone of inhibition = resistant
8	Loop of 4 hr. growth on Penassay Agar	Streptomycin Assay Agar	Plate dilution of antibiotics	Overnight (18 hr.)	Presence or absence of growth at each concentration	No interpretation. Reported as read, e.g., sensitive to 10 μg of antibiotic
9	One loopful of a 1:10 dilution of a 4-6 hr. growth in B.H.I. broth	Human Blood Agar	Bacto Disks*: 1 concentration	Overnight (18 hr.)	Size of zone of inhibition	Size of zones related to tables derived from previous tube dilution standardization are used to interpret zone sizes as sensitive, moderately sensitive, or resistant

*Difco Laboratories, Detroit, Mich. †Consolidated Laboratories, Inc., Chicago, Ill. ‡National Bio-Test Inc., Omaha, Neb. §Baltimore Biological Laboratory, Baltimore, Md.

Proteus vulgaris, and *Pseudomonas aeruginosa*. The strains were selected to obtain variable antibiotic sensitivities. Personal delivery of the cultures to each of the 9 laboratories insured the quick receipt of the specimens, encouraged prompt testing, and permitted first-hand knowledge of subtle variations in methodology in the individual laboratories.

Before distributing the organisms the sensitivity of each strain for various antibiotics was determined using two modifications of the serial twofold tube dilution method.[5] In one, a heavy bacterial inoculum (1:100 dilution of an 18-hour culture) was added to the test dilution of antibiotic; in the other, a light inoculum (1:10,000 dilution) was used. The minimal inhibitory concentration (M.I.C.) was determined by the absence of gross visual turbidity following incubation for 18 hours. Within 2 weeks after distribution of the organisms, they were collected from the individual laboratories, and the antibiotic sensitivities of many of the strains were repeated by the tube dilution method.

To avoid confusion caused by different systems of reporting the results of disk tests, the data were translated into one of four categories: sensitive, moderately sensitive, moderately resistant, or resistant. Four similar categories were established for the M.I.C. in the tube dilution tests as follows: 0-1, > 1-10, > 10-32, and > 32 μg/ml. In the comparison of the results by disk and tube dilution methods, only major discrepancies were considered different. Variations of plus or minus one category, a difference of about eightfold, were disregarded. Thus, the results of the two methods were considered incongruous only if a strain was found sensitive by a disk test and was not inhibited by 10 μg/ml. of antibiotic in broth, or moderately sensitive and not inhibited by 32 μg/ml. Also, conversely, if a strain was reported resistant by a disk test but inhibited by less than 10 μg/ml. of antibiotic or was moderately resistant and inhibited by less than 1.0 μg/ml. in the tube dilution test, the results were considered different.

RESULTS

Many differences were noted in the methods of disk sensitivity tests used routinely by the 9 hospital bacteriology laboratories. The details are shown in Table I. The strength and age of the inoculum varied markedly. Five different kinds of media, variable incubation periods, and 1 to 3 disks with different concentrations of the antibiotic were used. The latter were commercial disks which were manufactured by 4 different concerns. Five laboratories observed only the presence or absence of a zone of inhibited bacterial growth; 3 laboratories measured the size of these zones. Some bacteriologists looked carefully for a single resistant colony within the zone of inhibition, or for a thin film of spreading proteus; such findings were interpreted as bacterial resistance, while others ignored these findings and read the same results as indicative of bacterial sensitivity. Three laboratories reported their results objectively for interpretation by the clinician, but 5 laboratories interpreted their results in qualitative statements about the probable effectiveness of an antibiotic against the organism. The interpretations were based on directions supplied by the manufacturers of the disks, experience compiled by the laboratory directors, or data from the medical literature.

The frequency distribution of the results of sensitivity tests upon 25 standard strains with regard to 5 antibiotics is given in Table II. The minimal inhibitory concentrations determined in the tube dilution test with a heavy bacterial inoculum were usually one or two serial twofold dilutions greater than if a light inoculum was used. With the former the strains were inhibited by less than 10 μg per milliliter in 25 per cent of the tests. If a light inoculum was used this concentration was inhibitory in 53 per cent of the tests.

One hundred and thirty-six tube dilution sensitivity tests were repeated on the original cultures that were returned from the hospital laboratories. The results of 115 tests or 85 per cent, agreed within one twofold dilution of the original determination and 131 agreed within two serial dilutions. Thus, less than 4 per cent of the results of tube dilution tests performed at diverse times upon various bacterial species with several antibiotics varied more than fourfold. By the criteria of difference subsequently applied in the comparison of the disk and tube dilution methods, these results represent 100 per cent reproducibility.

TABLE II. ANTIBIOTIC SENSITIVITY OF THE STANDARD TEST STRAINS DETERMINED BY TWO MODIFICATIONS OF THE TUBE DILUTION METHOD

M.I.C.*	PER CENT OF STRAINS	
	HEAVY INOCULUM	LIGHT INOCULUM
> 32 μg/ml.	42	27
> 10-32 μg/ml.	33	20
> 1-10 μg/ml.	17	37
0-1 μg/ml.	8	16

*Minimal inhibitory concentration.

TABLE III. COMPARISON OF DISK AND TUBE DILUTION ANTIBIOTIC SENSITIVITY TEST RESULTS

DISK TEST RESULTS	MINIMUM INHIBITORY CONCENTRATION BY TUBE DILUTION TEST							
	0-1 μG/ML.		> 1-10 μG/ML.		> 10-32 μG/ML.		> 32 μG/ML.	
	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
A. Heavy Inoculum in Tube Dilution Test								
Resistant	0	0	21	13	107	33	277	68
Moderately resistant	0	0	21	13	53	17	51	12
Moderately sensitive	5	6	34	21	65	20	41	10
Sensitive	77	94	86	53	97	30	42	10
Totals	82	100	162	100	322	100	411	100
B. Light Inoculum in Tube Dilution Test								
Resistant	3	2	90	25	92	46	220	84
Moderately resistant	6	4	66	18	34	17	19	7
Moderately sensitive	17	11	83	23	32	16	13	5
Sensitive	127	83	124	34	41	21	10	4
Totals	153	100	363	100	199	100	262	100

A comparison of the survey results of the disk tests reported by the hospital bacteriology laboratories with those obtained by the tube dilution tests for the same strains and antibiotics is presented in Table III. The disk tests tended to show the strains to be more sensitive than the results of the tube tests with the heavy inoculum. Twenty per cent of the disks tests were reported as showing the strains to be sensitive or moderately sensitive when the minimal inhibitory concentration by tube dilution was greater than 32 μg per milliliter; one-half of the disk tests were similarly reported when the M.I.C. in the tube dilution test was greater than 10 μg per milliliter. These discrepancies were less marked when the disk results were compared with tube dilution tests in which a light inoculum was used. By this standard, however, 6 per cent of the disk tests indicated that the strains were resistant or moderately resistant when the M.I.C. was less than 1.0 μg per milliliter.

In Table IV the data are arranged to indicate the frequency distribution of different minimal inhibitory concentrations related to each category of results by the disk test. The heavily inoculated tube dilution test was the standard of reference. Strains reported as sensitive by the disk test corresponded to an M.I.C. of less than 1 μg per milliliter in 50 per cent and less than 10 μg per milliliter in 78 per cent of the observations. On the other hand, 22 per cent of the disk tests that reported the strains as sensitive were instances in which the M.I.C. was greater than 10 μg per milliliter. This was true for 53 per cent of the strains reported as moderately sensitive. At the other end of the scale, disk tests that reported the strains as resistant included 11 per cent in which the M.I.C. was less than 10 μg per milliliter.

TABLE IV. FREQUENCY DISTRIBUTION OF THE RESULTS OF DISK ANTIBIOTIC SENSITIVITY TESTS IN RELATION TO THE INHIBITORY CONCENTRATION BY TUBE DILUTION

M.I.C. OF TUBE DILUTION TESTS*	PER CENT OF STRAINS†			
	SENSITIVE	MOD. SENSITIVE	MOD. RESISTANT	RESISTANT
> 32 μg/ml.	5	17	29	60
> 10-32 μg/ml.	17	36	40	29
> 1-10 μg/ml.	28	37	31	11
0-1 μg/ml.	50	10	0	0
Totals	100	100	100	100

*Tube test with heavy inoculum.

†The number of strains in the various tube dilution categories in the original data has been corrected bringing them to a common denominator in each category.

Fig. 1 shows significant variations in the survey results of disk tests with the same organisms and antibiotics by different hospital laboratories. The results obtained by the tube dilution methods are again used as reference standards for comparison. Major differences in the results reported by any two individual laboratories were observed in as many as 25 per cent of the tests.

Fig. 2 illustrates the relationships of the individual antibiotics to major discrepancies between the two types of tests. The frequency distributions of the survey results by disk tests are shown for each category of sensitivity in the tube dilution test for each antibiotic. In the case of chloramphenicol and streptomycin the major discrepancies almost always showed the strain to be more sensitive by the disk test than by tube dilution. With polymyxin B the results of the disk test tended to indicate more resistant strains. With the tetracyclines major discrepancies were observed in both directions especially with strains of intermediate sensitivity with the strongest trend toward "false" resistance. Very few tests were done with strains of intermediate sensitivity to erythromycin or penicillin, and from these data it is difficult to evaluate the reliability of sensitivity tests with those two antibiotics.

Fig. 3 demonstrates major discrepancies in the results of disk and tube dilution antibiotic sensitivity tests as they were related to individual species of bacteria. The magnitude and direction of these discrepancies varied. The percentage of disk tests that showed the species to be more sensitive than by tube test was greatest for *Esch. coli*, 29 per cent, and least for *Ps. aeruginosa*,

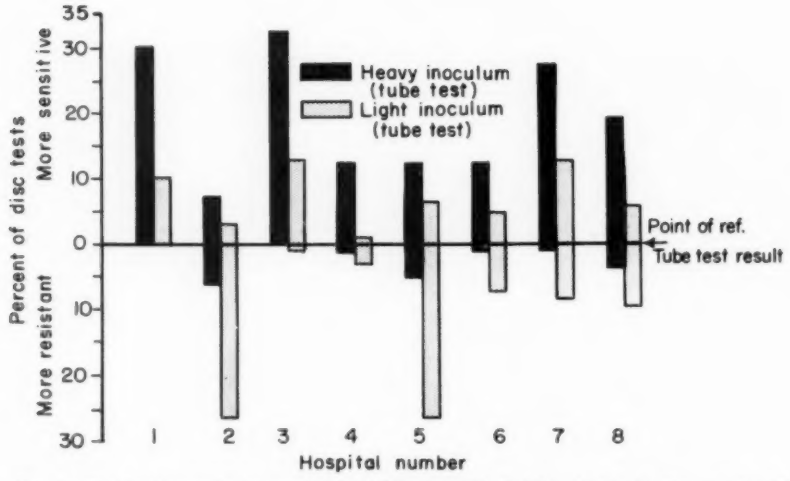

Fig. 1.—Major discrepancies among 8 different hospital laboratories in the results of disk tests for antibiotic sensitivity. Two modifications of the tube dilution method are used as a point of reference which shows the relative results obtained by the disk and tube methods.

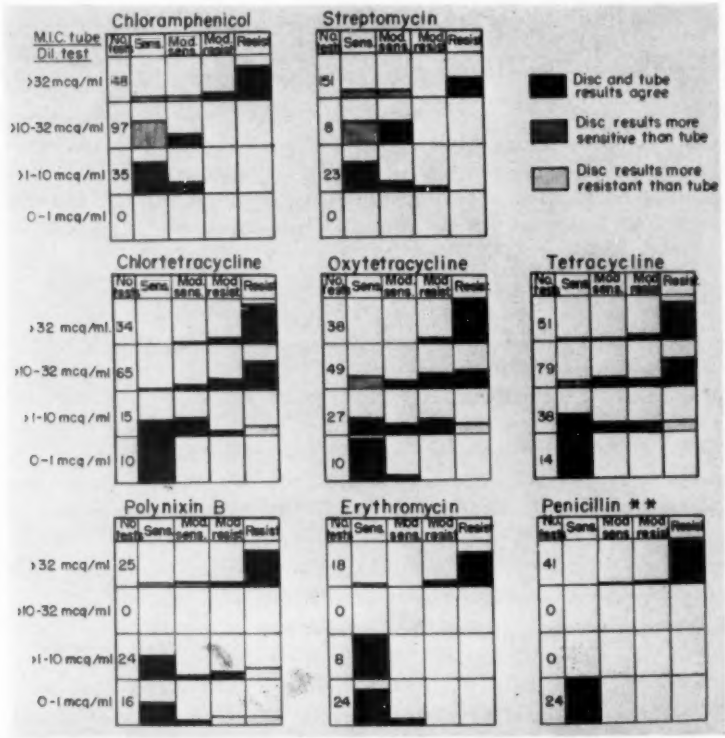

Fig. 2.—Frequency distributions of the results of the tube dilution sensitivity of standard strains to individual antibiotics in relation to the results observed by the disk method.

12 per cent, and *M. pyogenes* var. *aureus*, 15 per cent. *Ps. aeruginosa* and *A. aerogenes* were the only species in which some strains appeared significantly more resistant by disk test than by tube dilution.

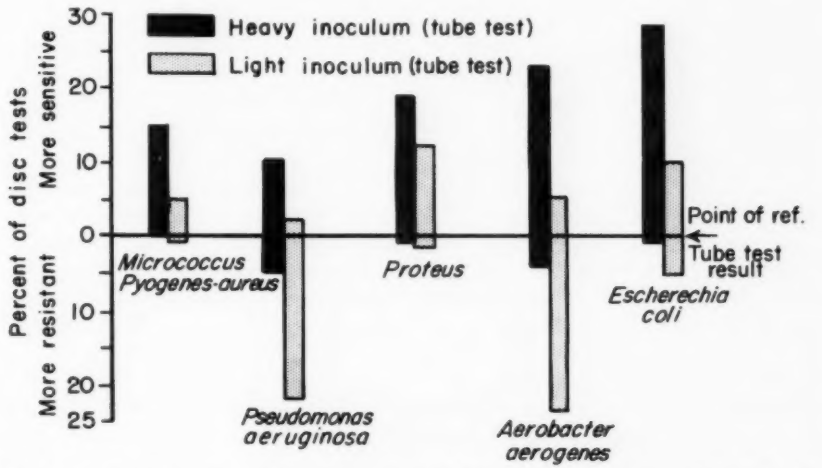

Fig. 3.—Major discrepancies in the results of disk and tube antibiotic sensitivity tests for different bacterial species.

DISCUSSION

Numerous reports in the medical literature[6-11] and our own experience indicate that many physicians are greatly influenced by the results of commercial disk antibiotic sensitivity tests in their choice of antibiotics for the treatment of patients with bacterial infections. Jackson,[4] Gould,[12] Branch,[14] and Neter,[13] and their respective co-workers, as well as others have demonstrated that the results of antibiotic sensitivity tests are affected by many factors, including: inoculum size; age of culture; pH, composition, thickness, and hydration of the media; incubation time; and the kind of commercial disks. Welch[15] has published an editorial regarding the demonstration by Cooper[16] and other investigators[17] that various antibiotics diffuse at different rates into agar media. Therefore, the size of the zone of inhibition cannot be used as a valid comparison of the relative effectiveness of various antibiotics.

The present survey supports the lack of standardization of methods in routine use to test the antibiotic sensitivity of bacterial strains[18, 19] and shows the variability in the results reported from different laboratories. Even the interpretation of the observed results of disk tests is not uniform. As reported by Branch and associates[20] different investigators do not agree on which disk concentration should be considered as "sensitive." Thus, any interpretation of a disk test result is an opinion rather than an established fact. It is obvious that the sensitivity value is absolute only for a well-defined environment and bacterial population.

Although the ideal control of infection in patients is the goal of these tests, the variability and complexity of clinical infections and pharmacologic factors in their treatment make it very difficult to use clinical data as convincing evidence of the reliability of disk tests for antibiotic sensitivity. For example, Broom and Martineau[21] reported good correlation between the results of disk tests and the results of treatment with various antibiotics, but their patients also received sulfadiazine.

In the present discussion the results of tube dilution tests were used as a standard of reference for the comparison of data obtained in the survey of individual laboratories. In the tube dilution method a defined concentration of an antibiotic and medium are used in which to measure the antibiotic effect. The use of a heavy bacterial inoculum in such a test is believed to most nearly represent the conditions of clinical infection and, therefore, the most reliable in vitro test. To broaden the basis for comparison, however, a second tube dilution test with a light inoculum was used. The results of repeated tube dilution tests which agreed within fourfold in 96 per cent of the tests represents the best agreement that could have been expected in the survey. This is less accurate than the results of repeated tube dilution tests reported by some investigators under different conditions.[4, 22] Nevertheless, the foregoing differences are negligible within the general categories established for this analysis.

The criteria for establishing a difference between the results of a qualitative disk test and a quantitative tube dilution test were necessarily very lenient; still, major discrepancies occurred in 20 per cent of the tests. These data suggest that disk tests generally err toward indicating increased sensitivity as compared to a tube dilution test with a heavy bacterial inoculum. The results with disk tests more nearly resemble those obtained in a tube dilution test with a light inoculum, although by this standard 17 per cent of the tests showed major discrepancies. Either type of test might make a strain appear more or less sensitive than the other as the factors in the methods of each test are varied. The disk test may err toward showing increased sensitivity and this appears to be the case for certain antibiotics in the concentrations now used in commercial disks. Other agents improperly appear less effective on a unit basis than should be the case. Freedom from this need for predetermined equation of drug concentrations is another recommendation for the tube dilution method.

Comparisons of the results of disk tests reported from the individual hospital laboratories, for various antibiotics, and for different species of bacteria indicated a significant degree of nonuniformity. The significant variation between different hospitals was anticipated because of the variation in methods. Among the individual antibiotics, the relatively large number of unduly sensitive results of disk tests with chloramphenicol and streptomycin was probably a reflection of the rapid diffusion and the bacteriostatic nature of the former and the marked variation in the sensitivity of individual clones to the latter. If the presence of one or two colonies within the zone of inhibition around a streptomycin disk was interpreted as a manifestation of resistance, the results approximated those obtained by tube dilution. Disk

tests with the tetracycline analogues and polymyxin B suggest "false" resistance in some tests; this was probably related to the rates of diffusion and deterioration of these drugs.[23] Among the individual bacterial species the per cent of strains of *Esch. coli* reported as more sensitive by disk than tube test was nearly double that of *M. pyogenes* var. *aureus*, while strains of *Ps. aeruginosa* sometimes appeared unduly resistant. The latter discrepancy was caused in part by two laboratories that reported 4 strains of *Ps. aeruginosa* resistant to polymyxin B. They were inhibited by a low concentration in the tube test, and to the best of our knowledge all strains of *Ps. aeruginosa* are sensitive to polymyxin B under these conditions. This antibiotic diffuses poorly into agar and may not give a good zone of inhibition with the disk test.

Collectively the data support the opinion expressed by Long[24] that a single in vitro disk test for antibiotic sensitivity is no more reliable than an educated guess. Results that show sensitivity of the strain should not be too readily accepted, particularly if they contradict the established values of antibiotic sensitivity for various species recorded in the literature. The results of disk sensitivity tests may be useful in screening species of bacteria that show wide variation in the antibiotic sensitivity of individual strains. Their reliability as an in vitro test to predict an in vivo effect is doubtful when the results vary so significantly with variations in the method of study.

SUMMARY AND CONCLUSIONS

A survey study of commercial disk antibiotic sensitivity test methods in 9 major teaching hospitals in Chicago has demonstrated that a wide variety of modifications of this method are in routine use. Significant variations were observed in the results of disk sensitivity tests of the same organisms and antibiotics by different hospital bacteriology laboratories. Comparison of the results by a tube dilution test with those reported by the survey disk tests suggested that the results of the latter generally tended to indicate greater sensitivity of the strains. This was more often true with chloramphenicol and streptomycin than with polymyxin B and the tetracycline analogues which sometimes showed the contrary effect. Discrepancies between the results of disk and tube tests also occurred in relation to different genera of bacteria. Over-all, major discrepancies between the disk and tube dilution methods were observed in 20 per cent of the tests. Within the limits used here for comparison the tube dilution test was uniformly reproducible. These findings demonstrated the difficulty in the standardization and control of disk antibiotic sensitivity test methods. The results show that there are doubtful benefits from the routine use of disk sensitivity tests, and indicate the need for caution in the interpretation of the results with regard to therapeutic measures. Antibiotic sensitivity tests are still considered less important criteria for the selection of a proper antibiotic for the treatment of bacterial infections than the clinical diagnosis, isolation of the causative organisms, and knowledge of the properties of specific antibiotics.

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THE SEROLOGY OF MUMPS INFECTIONS

I. A New Source of Antigen and a Simplified Complement Fixation Test

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WHEN a diagnostic laboratory is called upon to perform both screening tests and quantitative titrations at a moment's notice, and the number and frequency of such requests is unpredictable, it is soon realized how wasteful of materials and time the orthodox serologic methods can be. With an improvement of these in mind, we started a systematic study into the production and storage of the necessary reagents, and their most satisfactory use. The techniques to be described have now been in use for over 5 years, and should prove particularly suited for laboratories with no access to reagents produced on a commercial scale, and which lack expensive physical equipment for the purification of proteins.

The present paper reports on the complement fixation test with the small (or "soluble," nonhemagglutinating) antigen, a method found best for the recognition of mumps virus infections of diverse clinical appearance. A new, rich source of this antigen will be described, as well as a technique which, although of sufficient accuracy, does not involve tedious daily standardization of reagents, the bugbear of complement fixation tests.

MATERIALS

Calcium-magnesium saline was prepared by dissolving 8.50 Gm. NaCl, 0.028 Gm. $CaCl_2$, and 0.079 Gm. $MgCl_2$ in 1,000 ml. of 0.02 M borate buffer at pH 7.2. The solution was sterilized by autoclaving at 120° C. for 20 minutes. *Gelatine saline*, used as diluent in infectivity tests, contained 5.0 Gm. pure gelatine in 1 L. of calcium-magnesium saline.

Red blood cells from fowls, sheep, or humans were collected in Alsever's solution, washed 3 times by spinning through about 10 volumes of saline, packed, and stored at 2° C.

Mumps virus was grown from stocks of glycerolated seed (30 per cent glycerol v/v) kept at −20° C. Usually 0.1 ml. of a 10^{-3} dilution in gelatine saline was inoculated amniotically into a group of 9-day embryos. The amniotic fluids were harvested aseptically after 5 days' incubation at 36° C., and stored either at 2° C. for shorter periods of time, or ampouled, snap-frozen, and kept at −70° C.

The Enders strain, the prototype of mumps virus, was used in most experiments. Our subculture has been passed repeatedly in eggs, and grew equally well in the amnion and allantois. The other strain, 3DB, was isolated in 1952 at the Royal Children's Hospital, Melbourne, from the saliva of a child admitted with aseptic meningitis. This virus had been through two amniotic and one allantoic passage, but showed only patchy growth in the latter system.

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Antisera collected from patients convalescent after mumps parotitis or meningitis were stored in the frozen state (−10 or −20° C.), without any preservative.

The technique of complement fixing and hemagglutinin tests followed closely the description of Henle, Henle, and Harris[1] in the earlier parts of this study; the methods developed later are given in full in the concluding section of this paper.

METHODS

Preparation of Antigen.—From the fundamental work of Henle, Henle, and Harris[1] it was known that most of the small (*S*) antigen stayed in the tissues infected by mumps virus, while the infective particles were found predominantly in the extraembryonic fluids. No clear indication was given however, which of the possible sources was the richest, what the optimal infective dose was, and at what age of incubation were the eggs most suitable for the production of *S* antigen. Also, it seemed possible that other routes of inoculation, or other tissues besides the allantoic and amniotic membranes might give better results.

Route of Inoculation.—In a sighting experiment, groups of ten 9-day eggs were inoculated with approximately 10^3 HD_{50} of mumps virus (Enders strain) into the allantoic cavity, amniotic cavity, yolk sac, or onto the chorion. After 6 days' incubation the allantoic and amniotic fluids, the chorioallantoic and amniotic membranes, the yolk sac, the embryo and, separately, the embryonic lungs* were harvested from each egg. Equal amounts of the 10 replicate samples were pooled, and the tissues were ground with alundum, made up to a 20 per cent suspension (wet weight) in saline, and spun at about 1,500 g for 5 minutes. The extraembryonic fluids and the supernatants of ground tissues were then tested for hemagglutinin content. The titers obtained give a measure of the *V* antigen present, and appear under this symbol in Table I. Then, to remove the bulk of virus which might interfere in the complement fixation test for the *S* antigen, an aliquot of each pool was absorbed with human red blood cells. One-tenth, by volume, of packed red cells was added to the chilled fluids; the mixtures were well shaken and left at 4° C. for 30 minutes. The cells were then spun down, and the procedure repeated on the supernatant. The efficiency of absorption was checked in a second hemagglutinin test. As will be seen from the titers after absorption (figures in parentheses in Table I), the method was not invariably successful. Whereas in 4 samples 90 per cent or more of the hemagglutinin could be removed, in one about a sixth and in another about a half was left behind. These latter pools of allantoic fluid were murkier than the rest, due to some contamination with yolk. Since however, the residual virus did not interfere in the complement fixation tests, the matter was not followed further.

In testing for the *S* antigen one hour at 37° C. was allowed for fixation of complement, using a human convalescent serum. This serum showed high titers

*The method found simplest is as follows: The embryo is laid, back up, on a pad of moist cotton wool, and held down by grasping the skin between the shoulder blades with a pair of small surgical forceps. The spine is cut through at the level of the first thoracic segment, and the cut is widened by running through the ribs at an angle of about 45°, on each side. Directly under this triangular flap lie the lungs; in most cases they can be lifted out without severing the mediastinal structures.

against both the *S* and *V* antigens, and thus its use could be questioned on account of its double specificity. This theoretical objection was not supported by the results: the 3 fluids with some residual hemagglutinin after adsorption gave complete lysis even at the lowest dilution tested (1:2). This finding is in line with the original observations of Henle and associates[1] who obtained no complete fixation of complement with the *V* antigen if a high-titer allantoic fluid was diluted beyond 1:6. On these grounds the titers in our complement fixation tests are regarded as specific for the *S* antigen.

TABLE I. THE YIELD OF *V* AND *S* ANTIGENS AFTER INOCULATION OF 9-DAY CHICK EMBRYOS BY DIFFERENT ROUTES WITH 10^3 ID_{50} OF MUMPS VIRUS

SPECIMEN TESTED*	ROUTE OF INOCULATION							
	ALLANTOIS		AMNION		CHORION		YOLK SAC	
	V	*S*	*V*	*S*	*V*	*S*	*V*	*S*
Allantoic fluid	150 (20)†	–	110 (–)	–	160 (–)	–	65 (30)	–
Amniotic fluid	–	–	50 (5)	–	–	–	120 (–)	–
Chorioallantois	–	8	–	4	–	7	–	16
Amnion	–	6	–	32	–	5	–	32
Yolk sac	–	6	–	7	–	6	–	20 (Ac at 1:8)
Lungs	–	–	–	44	–	–	–	40
Embryo	–	Ac	–	Ac	–	Ac	not tested	

Minus sign (–) = <5 in hemagglutinin tests, and <2 in complement fixation tests.
Ac = anticomplementary.

*The titers refer to undiluted fluids or to the supernatants of 20 per cent tissue suspensions.

†The figures in parentheses are the titers after absorption with human erythrocytes.

The important finding emerging from this experiment is the high yield of *S* antigen from the embryonic lungs after amniotic inoculation of virus. They contained more *S* antigen than any other tissue, the only comparable source being the amniotic membrane. Indeed, these two represented more than three-fourths of the output of the eggs after inoculation by the two routes which ensured their infection.

Although inoculation into the yolk sac is technically the simplest, 7 out of 10 eggs receiving mumps virus by this route died within 3 days. As a similar rate of mortality was observed in another test, this route was not used in later experiments, especially since the yields after amniotic infection were equally high and unaccompanied by the death of the embryo.

Age of the Embryo and Length of Incubation.—Following up this lead, a set of factorial experiments was designed paying special attention to the antigen content of the lungs. Since, unlike the extraembryonic membranes, the lungs show progressive development right up to the time of hatching, it seemed likely that in older eggs the larger mass of lung tissue would contain more *S* antigen. Thus groups of 20 eggs were inoculated by the amniotic route at the age of 10, 11, 12, 13, and 14 days, respectively. They received $10^{1.8}$ ID_{50} of the Enders strain in a volume of 0.10 ml. Five eggs of each group were taken down after 4, 5, 6, or 7 days of incubation at 35° C., and the amniotic

fluids and lungs of each embryo harvested. The lungs were ground, made up to a 20 per cent suspension, and spun at low speed. The supernatants of the lungs as well as the amniotic fluids were titrated individually for hemagglutinin, and then absorbed with two changes of 10 per cent human red blood cells. After absorption the fluids were tested for *S* antigen by complement fixation. The results, in the form of subgroup averages referring to 5 eggs each, are given in Table II.

TABLE II. THE EFFECT OF AGE AND LENGTH OF INCUBATION ON THE YIELD OF *V* AND *S* ANTIGENS AFTER AMNIOTIC INOCULATION OF $10^{4.8}$ ID_{50} OF MUMPS VIRUS

AGE OF EMBRYO		TITER OF:			
		AMNIOTIC FLUID		LUNGS*	
AT INOCULATION	AT HARVEST	*V*†	*S*‡	*V*†	*S*‡
10	14	19	1	4	1
	15	79	1	–	3
	16	181	4	–	8
	17	97	–	–	26
11	15	12	–	–	8
	16	84	2	–	58
	17	160	1	–	27
	18	74	–	–	7
12	16	43	–	4	4
	17	114	1	–	24
	18	84	2	–	90
	19	27	–	–	22
13	17	3	–	–	6
	18	43	–	–	58
	19	56	–	–	121
	20	63	1	–	56
14	18	17	–	–	2
	19	21	–	–	5
	20	5	–	–	8

*Supernatant fluids of 20 per cent tissue suspensions (w/v).
†Hemagglutinin titers.
‡Complement-fixing titers.
Minus sign (–) = titer < 3 in hemagglutinin tests, and < 1 in complement fixation tests.

As is usual with mumps virus, the samples within each group showed considerable scatter in titers, fourfold differences between the extremes being not uncommon. The averages are, however, still reliable enough to suggest some general trends. Thus it appears that, with the inoculum used, 5 to 7 days is the optimum period of incubation; 6 days is perhaps somewhat better than either 5 or 7. As regards the age at which the embryos should be inoculated, there is a definite peak in the 11-, 12-, and 13-day groups, both younger and older embryos showing significantly lower yields. The virtual absence of hemagglutinating *V* antigen from the lung extracts is evident once more.

The behavior of mumps virus here seems analogous to that of 0-phase influenza viruses which are released into the amniotic fluid after rapid multiplication in the lungs.[2, 3] A fortunate occurrence, this phenomenon enables us to use the embryonic lungs as specific antigen without the need of previous removal of contaminating virus by adsorption onto red cells or by high-speed centrifugation. (It should be added that in some of the later experiments, using a local strain of mumps virus, a few lungs were found to contain both

V and *S* antigens. The hemagglutinin content of these preparations was always very low, and although specially tested for, it was never found to interfere in complement fixation tests. Thus, for routine serologic purposes, the lung extracts may be regarded as suitable antigens without any further purification.)

Preliminary Screening of Lungs.—In the early experiments we were struck by the observation that although the lungs contained no hemagglutinin, the corresponding amniotic fluids did. Clearly, if this relationship held regularly, it would afford a simple presumptive test: if a drop of amniotic fluid agglutinated a suspension of red cells, it could be assumed that the respective lungs were of satisfactory *S* antigen content. The results of such a test would be available at the time of harvesting the lungs.

However, the correlation was not strict. Although statistical evaluation of 262 paired tests gave a χ^2 of 14.95 (and thus a probability of <0.001), in about 28 per cent of all cases the answer was incorrect; in some instances hemagglutinating amniotic fluid was found with no complement-fixing antigen in the lungs or, more often, negative spot tests with positive lungs. Such a margin of error is too wide, and consequently the preliminary screening of lungs by this method is of no practical use.

Size of the Infective Dose.—The next experiment served to establish the optimal virus inoculum. Groups of 12-day eggs were inoculated amniotically with the Enders strain of mumps virus in doses ranging from $10^{0.5}$ to $10^{7.5}$ ID_{50}. After different periods of incubation at 35° C. 5 eggs of each group were taken down. A sample of amniotic fluid was tested for hemagglutinin, and the lungs removed, ground, and suspended in the usual manner. However, the extracts were not absorbed with red cells, as it was known by now that such preparations contained little or no detectable hemagglutinin, and what there was of it did not interfere with the assay of *S* antigen. The *S* antigen titers of embryonic lungs are given in Table III, each figure showing the geometric mean of the eggs found alive on the day indicated.

TABLE III. THE YIELD OF *S* ANTIGEN FROM EMBRYONIC LUNGS AFTER AMNIOTIC INFECTION WITH VARIOUS DOSES OF MUMPS VIRUS

INOCULUM (ID_{50})	LENGTH OF INCUBATION (DAYS)			
	4	5	6	7
$10^{0.5}$	3.6	48.5	76.1	238.8
$10^{1.5}$	8.9	55.0	36.8	326.3
$10^{2.5}$	26.0	29.9	93.7	401.6
$10^{3.5}$	70.0	90.5	200.8	123.6
$10^{4.5}$	65.0	284.1	90.5	147.1
$10^{5.5}$	97.0	190.0	181.0	137.2
$10^{6.5}$	181.0	130.0	170.0	84.5
$10^{7.5}$	102.0	137.2	80.0	17.2

The figures give the geometric mean titer of the survivors from a group of 5 eggs.

The first impression gained from Table III is that the size of the initial dose makes little difference to the yield of *S* antigen. The peak titers are of the order of a hundred in each group, although with the 3 smallest inocula it

takes about 6 days to reach this level, whereas the 3 largest have attained it by the fourth day. The falling off after the sixth day, noticeable in the two highest groups, is probably significant.

What the figures do not show is the poor survival of embryos after high infective doses of mumps virus. With inocula up to $10^{5.5}$ ID_{50} not more than about 1 in 20 of the embryos died per day. The 4 largest doses often killed half of the chicks by the fourth day, and practically all by the seventh. The lungs coming from dead embryos were also tested separately and found to contain usually as much antigen as did live lungs. However, their harvesting was often difficult because of partial autolysis, and many of them were found to be anticomplementary. For these reasons the optimal starting dose of virus lies between 10^2 and 10^4 ID_{50}, since in this range all embryos are infected, only few of them die, and the lungs reach uniformly high titers between the fifth and seventh day of incubation.

The experiments detailed above, as well as others in which the same factors were tested in different combinations and over different ranges, defined the conditions for the optimal production of mumps complement-fixing antigen. Accordingly, 11- to 13-day eggs should be inoculated amniotically with 10^2 to 10^4 infective doses of virus and incubated for 5 to 6 days. The highest concentration of *S* antigen is to be found in the embryonic lungs. This source has the added advantage of containing only such small amounts of the hemagglutinating *V* antigen as will, for the most part, escape detection, and even if detectable will not interfere with the test for the small antigen.

The Effect of Strain Differences.—To establish these conclusions on a wider basis and to test at the same time the effect of strain differences, the next experiment was performed on a local strain of mumps virus. All factors found important in the earlier experiments were tested over a range extending in both directions beyond the optimum found with the classical strain of mumps virus. Thus eggs 9 to 14 days of age received $10^{2.5}$ or $10^{4.5}$ ID_{50} of the 3DB strain amniotically. After 2 to 6 days' incubation the allantoic and amniotic fluids of each egg, as well as suspensions of the chorioallantois, the amnion, the embryonic lungs, and a pool of other tissues (skin, muscle, heart, liver) were tested both for hemagglutinin and for complement-fixing antigen by the standard method. As the tissues were taken up in the same volume of saline (1.0 ml.), the titers are proportional to the total content of antigen rather than to its concentration in that particular tissue. In the histograms of Figs. 1 to 5 the geometric means are shown (*S* antigen in black, *V* antigen in white), each column referring to 5 embryos.

The embryonic lungs (Fig. 1) again stand out as the most consistently rich source of antigen. The optimum age (12 to 13 days) is the same as for the Enders strain, as is the period of incubation (5 to 6 days). Differences in yield due to inocula are not appreciable in this range, although the larger dose killed many embryos within 3 days, while $10^{2.5}$ ID_{50} had no such effect. The hemagglutinin content of these preparations was negligible throughout, and none was found to be anticomplementary.

The two strains differ in their ability to produce *S* antigen in embryonic lungs of different ages. The complete absence of *S* antigen from lungs of embryos inoculated with the 3DB strain at a younger age was in contrast to the results obtained with the Enders virus. This finding was the more perplexing as the corresponding amniotic fluids contained if anything, more virus than a similar group infected with the Enders strain (compare Table I and Fig. 4), and the amniotic membranes of the same embryos had higher *S* antigen titers

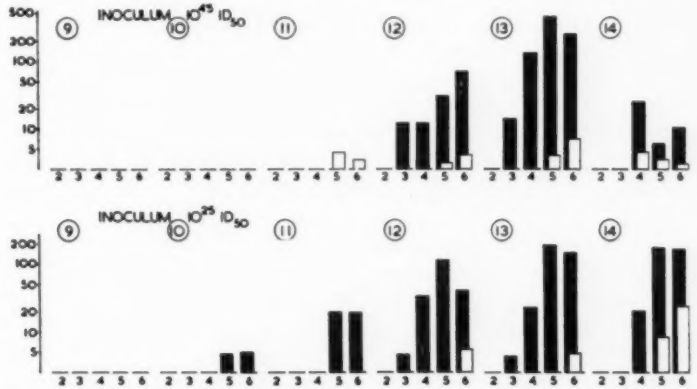

Fig. 1.—Yield of mumps antigens from embryonic lungs after amniotic inoculation of the virus. The encircled figures show the age of the embryo on the day of inoculation. The abscissa gives the length of incubation; the ordinate gives the titers. Each column refers to a group of 5 embryos: *black columns*, *S* antigen (determined by complement fixation); *white columns*, *V* antigen (determined by hemagglutination).

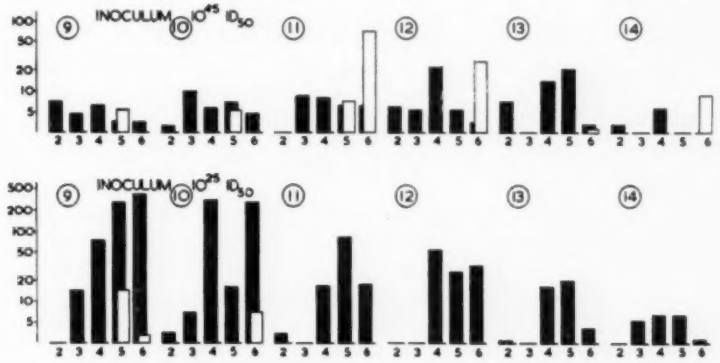

Fig. 2.—Yield of mumps antigens from amniotic membranes after amniotic inoculation of the virus.

than at any other age (Fig. 2). It is known that the pharyngeal membrane constitutes a physical barrier to the passage of amniotic fluid into the lungs up to the end of the second week of embryonic development, and this could perhaps explain why the smaller inoculum which multiplies over a longer period was capable of invading the lungs of some of the younger embryos.

But it cannot account for the difference between the Enders and 3DB strains, unless one supposes either that the former has a much higher rate of growth in lungs, or that the latter produces mainly noninfective or rapidly inactivated offspring, incapable of spreading to the lungs. This question, beyond the scope of this study, was not pursued and must remain unresolved for the present.

As regards the amniotic membranes, the difference due to inocula here is striking. After the larger dose, the yields are uniformly low over the age group tested, and there is little variation between harvests on different days. After the smaller dose, young embryos produce large quantities of *S* antigen, with the peak in the 9- to 10-day groups and a gradual decline thereafter. The optimal length of incubation is 4 to 6 days. The fluctuation within each group, however, was considerably wider than in lungs, and for this reason alone the membranes are a less dependable source of antigen for purposes of routine production. Another, more serious, drawback was the finding that 47 membranes (out of 138 positive) were anticomplementary at dilutions of 1 in 10 or higher.

The yield of *S* antigen from the chorioallantois was sporadic and altogether insignificant after the larger inoculum. After the smaller, titers were of the order described by Henle and co-workers[4] and, in agreement with their experience, younger eggs were superior to older ones (Fig. 3). A few membranes (11 out of 287) were found to be anticomplementary.

The extraembryonic fluids are of little interest as a source of *S* antigen, partly because of low and irregular yields, and partly because all positive fluids also showed hemagglutinin titers of an order that would render them unsuited for the testing of anti-*S* antibodies without previous removal of the *V* antigen.

When comparing the hemagglutinin titers of the amniotic (Fig. 4) and allantoic (Fig. 5) fluids, it should be remembered that the 3DB strain had had only one allantoic passage at the time of this experiment; it is the lack of adaptation that underlies its unpredictable growth in the allantois.

The extracts of pooled embryonic tissues had either no titers or were anticomplementary and, hence, of no practical value.

Storage of Antigen.—The pools of embryonic lung suspensions prepared by the optimal method gave complement-fixing titers ranging between 80 and 200. As each milliliter of such preparations is sufficient to perform tests on about a hundred specimens of serum (supposing 6 steps of dilution), the keeping qualities of the antigen are of importance. At first we stored the antigen at −10° C. in sealed ampoules containing 0.5 ml. of the lung extracts. Although ampoules belonging to one batch opened up at the same titer over a period of 8 months, the titer was about a tube lower than before freezing, i.e., about half of the crude antigen was lost in the process. Later, the titrated lung extracts were diluted with calcium-magnesium saline to the optimal concentration of antigen (5 units per standard volume), ampouled in 0.5 to 5.0 ml. volumes, and then frozen. In this form the antigen kept equally well and without any noticeable loss due to freezing and thawing. This latter method

of storage has the advantage that the content of the ampoules can be used directly on thawing, without preliminary titration, and that there is no waste since only the volume required need be opened. However, the storage of many ampoules is space consuming.

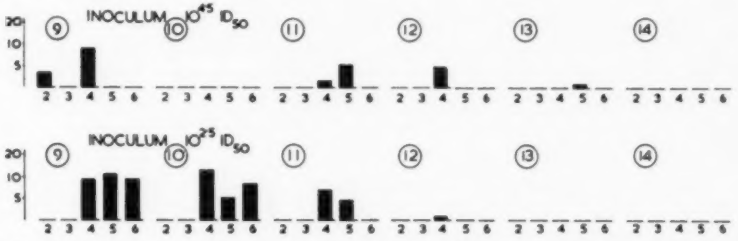

Fig. 3.—Yield of mumps antigens from the chorioallantois after amniotic inoculation of the virus.

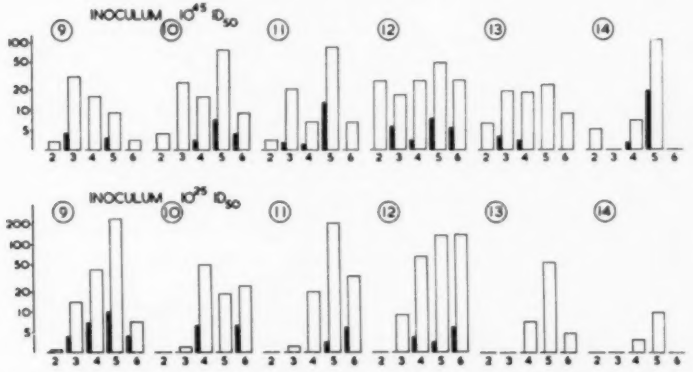

Fig. 4.—Yield of mumps antigens from amniotic fluid after amniotic inoculation of the virus.

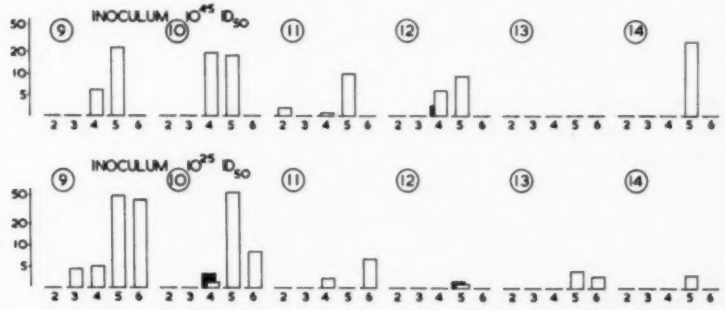

Fig. 5.—Yield of mumps antigens from allantoic fluid after amniotic inoculation of the virus.

Finally the method used by Hoyle[5] for influenza complement-fixing antigen was tried and found entirely satisfactory. The lungs are harvested, rinsed in saline, and then placed, without grinding, in a desiccator over anhydrous

$CaCl_2$. (It is not necessary to evacuate the jar.) After a day or two the dry lungs are collected, pooled, and ground without any additive in a mortar. The powder is transferred into a small vessel lightly stoppered with cotton wool and replaced in the desiccator. For use, the required quantity is weighed out and made up to a 1 per cent suspension in saline. After incubation, with repeated vigorous shakings, for 30 minutes at 37° C. the suspension is spun in an angle centrifuge, and the supernatant pipetted off. Such preparations have invariably higher titers than comparable ones made by grinding the lungs in the wet state, presumably due to the better dispersion afforded by dry grinding.

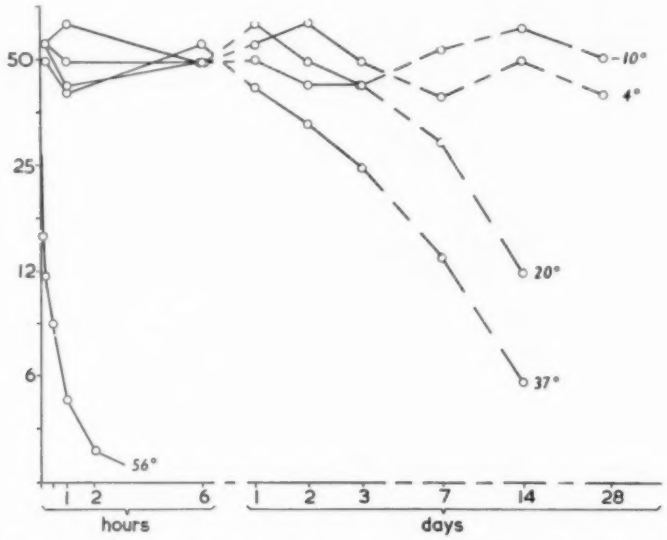

Fig. 6.—Inactivation of the *S* antigen by heat. The abscissa gives the time of exposure to the temperature (in degrees centigrade) shown on the respective curves; the ordinate shows the complement-fixing titer.

Extracts prepared in this way will keep unchanged for at least a fortnight if kept in the refrigerator, as shown by the following experiment in which their heat resistance was tested at 5 levels of temperature (Fig. 6).

At 56° C. the inactivation is rapid; at 37° C. the half-life is about 60 hours; and at 20, 4, and −10° C., respectively, the variation lies within the accuracy of the titrations. Thus it is feasible to store the antigen-containing lungs in the dry state for indefinite periods of time, and extracts prepared to cover a week's requirements can be kept conveniently and without loss of titer. Since such extracts can be made up reproducibly, preliminary titrations of antigen preceding each test become unnecessary.

Reagents and Technique of Standard Tests.—Apart from the antigen, the reagents employed in the complement fixation test, as well as the details of

technique, did not differ from what had been proposed by one or the other worker in the past. Since, however, there are numerous and at times conflicting recommendations, it might be as well to record briefly our experience in comparing these variants and to give reasons for preferring the combination which has become the standard method in this laboratory.

Antiserum.—As clinical cases of mumps occur mostly in children and the collection of large volumes of blood from convalescents is therefore not practicable, our positive control sera were lately prepared in guinea pigs. The method is essentially that of Sohier, Challut, and Chardonnet.[6] Healthy adult animals received about 10^4 to 10^6 infective doses of mumps virus, in the form of a 0.1 ml. inoculum into each nostril. After a lapse of 2 to 4 weeks a booster dose of 10^7 HD_{50} was given, again intranasally; the animals were bled 5 days after the second inoculation. At this time their sera fixed complement with the *S* antigen when diluted 1:200 to 1:800, and had an antihemagglutinin titer of the same order. The latter gave no cause for concern as the extracts of infected lungs contained too little *V* antigen to be even detected by the standard complement fixation test.

The sera were diluted 1:5 in saline, inactivated at 62.5° C. for 15 minutes, ampouled in 0.5 ml. volumes, and stored frozen at –10° C. In this state the titer remained unchanged, and the contents of one ampoule were sufficient to serve as controls in 10 to 12 sets of titrations.

Negative control sera of human origin were handled in the same fashion.

Complement.—A large pool of guinea pig serum was distributed in exactly 8.0 ml. lots, into small screw-cap containers and stored at –10° C. For use one of these was thawed and preserved by the addition of Richardson's solutions.[7] At first, complement was routinely titrated before each main test, both in the presence and absence of antigen. With time it became obvious that frozen storage preserves it without loss, and the thawed samples retained their titer unchanged for at least a fortnight at refrigerator temperature. Consequently, the preliminary titration of complement was abandoned, and instead the dose actually used in the main test was checked at dilutions of 1:1, 1:2, 1:3, 1:4, 1:6, and 1:8. Since we aimed at 3 HD_{50}, this set gave a complete series of reactions, and even minimal changes would have been detected if present.

Hemolytic System.—High-titer lytic serum against sheep erythrocytes was prepared in rabbits by a month's course of multiple subcutaneous injections of 0.5 ml. washed sheep red cells every 5 days. A week after the last injection the animals were bled, the sera separated, inactivated, ampouled in 0.5 ml. lots, and stored frozen at –10° C. The hemolytic titer of such sera was in the range of 3,000 to 5,000.

Sheep red cells were collected weekly and stored in the modified Alsever's solution at 4° C. Before sensitization the cells were washed 3 times by spinning through 10 volumes of saline, and were then made up to a 4 per cent suspension. Finally, 8 HD_{50} of hemolysin was added in an equal volume of saline, the mixture incubated at 37° C. for 30 minutes, and then transferred to the refrigerator. A fresh lot of cells was sensitized for each day's tests.

The Test.—Since the samples of blood sent to the laboratory were usually of small volume, the standard tests were so designed that a complete set of titrations could be performed on 0.05 ml. of serum. At first the tests were set up in small (10 by 0.8 cm.) test tubes; later transparent plastic trays, designed originally for hemagglutinin tests, were used. As there is some difference in the technicalities of these alternative methods, they will be described separately.

In the *tube-test* calibrated dropping pipettes (0.03 ml.) were used to make up dilutions. One standard drop of the serum to be tested was mixed with three drops of saline and inactivated at 62.5° C. for 15 minutes. This starting dilution was then halved. One set (6 twofold steps) was tested against the standard mumps antigen; the other (4 twofold steps) against the negative antigen prepared from uninfected embryonic lungs. This latter set also served to detect any anticomplementariness in the serum.

The positive antigen, made up to contain 4 units per standard drop, was mixed with an equal volume of complement diluted to a concentration of 3 HD_{50} per standard drop. Of this mixture two drops were added to each tube. The normal antigen, diluted to the same degree as the positive one, was similarly mixed with complement and two drops of it added to the set of control tubes. The racks were then shaken and placed in a water-bath at 37° C. for 45 minutes. In the "short fixation" test the sensitized cells were added at this stage; in the "long fixation" test the racks were transferred to an icebox for 16 to 20 hours, and then warmed in the bath for 10 minutes before receiving the standard drop of sensitized cells. Three-quarters of an hour was allowed for lysis, and the racks were shaken twice during this period.

Three intermediate degrees of turbidity were distinguished between complete and no lysis. The central reaction (50 per cent lysis) was taken as the end point.

In the *tray-test* the two sets of dilutions were made up with the aid of Takátsy's spiral loops,[8, 9] in a standard volume of 0.05 ml. This method is considerably quicker than dilution by drops, and at least as accurate. The mixture of antigen and complement was delivered with an automatic pipette, and the trays placed in an incubator or warm room (36° C.). It is important not to pile the trays on one another, as this prevents their uniform warming during the 45 minutes of incubation. Again, as in the tube-test, the hemolytic system can be added at this stage, or the trays can be transferred to the refrigerator. In the latter case it is convenient to arrange them in piles to cut down evaporation. The differential cooling here becomes unimportant, being relative to overnight incubation. Next day the trays are laid out individually in the incubator for 30 minutes, and then the hemolytic system is added. At this stage we placed the trays in a shaking machine (120 horizontal oscillations per minute, 2 cm. thrust) for an hour at 36° C. The results are the same after stationary incubation at 36° C., provided the trays are shaken at least twice during the period.

The test can be read in the ordinary way or, more conveniently, by letting the cells settle at room temperature for about an hour. Inspected from above against a white background, the positive cups contain a dense central button of red cells; intermediate degrees of lysis are characterized by smaller central buttons and correspondingly stronger pink color in the medium. In negative cups all cells are laked, and the fluid is uniformly colored, transparent, and without any trace of turbidity or deposit. These patterns do not change for at least two hours after the cells have settled and thus, unlike in the tube-test, reading of the results need not be strictly timed.

On the whole, we prefer the tray-test for its greater accuracy (see below), for the time saved in setting it up, and for the ease of cleaning the equipment. (After use the plastic trays are immersed in 4 per cent NaOH for an hour, thoroughly rinsed with running tap water and left to dry, face down, at room temperature.)

Reproducibility.—Experiments conducted under this heading sought to answer the following questions:

1. What is the variation between tests done simultaneously by one operator?

2. What additional error is introduced by several operators using the same materials?

3. How do tests compare when done on different days and/or with different reagents?

Altogether 4 factorial experiments were performed. In the first 2 of these, only the tube-test was used; the other 2 included also a comparison of the tube- and tray-tests. On analysis the results were found consistent enough to allow pooling of the information, the contrasts being represented by their mean contributions to the variance.

Thus, the standard deviation (standard error of a single titration) is ± 0.31 $\log_2$ unit (or 27 per cent) for the tube-test, and ± 0.23 $\log_2$ unit (or 19 per cent) for the tray-test. It is noteworthy that this error depends greatly on the operator in the tube-test (range: 0.22 to 0.47) and barely so in the tray-test (range: 0.20 to 0.26). In other words, whereas careful work leads to the same accuracy in both tests, the use of trays and spiral loops has fewer pitfalls.

The difference between operators was found insignificant in the tray-test; with tubes the variance ratio became just significant ($P \sim 0.04$). Tests done on different days, on the other hand, varied significantly whether performed with the same or with different reagents. The error of such tests was ± 0.48 $\log_2$ unit (or 44 per cent) and ± 0.43 $\log_2$ unit (or 40 per cent) for tubes and trays, respectively. The accuracy of antigen and antibody titrations proved to be the same.

It follows that when two sera are compared carefully on the same day by the same worker, a difference of 1.31 $\log_2$ units (2.7 fold) is significant at the

95 per cent level of probability. To tell apart, with the same degree of confidence, the results of two tests done in the same laboratory but at different times, a difference of 2.44 $\log_2$ units (6.5 fold) is required. This should be strong inducement to titrate side by side all specimens to be compared.

The order of accuracy achieved by the test is all that is needed in a diagnostic laboratory. If, for some special purpose, greater sensitivity and less variation are demanded, the method of choice would be the back-titration of residual complement after letting the test sample react with an excess of the other two reagents. A technique based on this principle has been adapted recently to the serology of mumps by Wigand.[10] Unfortunately, improved sensitivity is guaranteed only in two-dimensional tests, and these, even on a microscale, use extravagant volumes of serum; moreover, by its nature this method cannot work as a spot-test. Hence, it is less practical, at least in a children's hospital, than the more orthodox technique outlined above.

SUMMARY

A new source of mumps complement-fixing antigen is described. Chick embryos of 11 to 13 days' incubation are infected amniotically with 10^2-10^4 ID_{50} of virus. After 5 to 6 days at 36° C. their lungs are harvested, ground, and suspended in saline. Such preparations have titers 5 to 20 times higher than standard chorioallantoic antigens, are not anticomplementary, and are free from interfering viral antigens.

A complement-fixing technique, requiring 0.05 ml. of serum is given in detail, together with methods of preparing and storing all reagents required in the test.

The accuracy and reproducibility of the technique is evaluated statistically.

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THE EFFECT OF ORAL FEEDINGS OF MEAT AND GLUCOSE ON HEPATIC VEIN WEDGE PRESSURE IN NORMAL AND CIRRHOTIC SUBJECTS

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ONE of the most important factors in the genesis of splenomegaly, ascites, esophageal, gastric, and hemorrhoidal varices in patients with cirrhosis of the liver is portal venous hypertension. A rise in portal vein pressure probably plays an important role in the bleeding from varices in cirrhotic subjects. The causes of pressure changes in the portal venous circulation are, however, poorly understood.

Measurement of the hepatic vein wedge pressure (HVWP) accurately estimates portal vein pressure (PVP), and it has been shown at laparotomy in human subjects that HVWP is approximately 80 per cent of the portal vein pressure (PVP) and that change in PVP is reflected in HVWP.[2] This subject was recently reviewed by Reynolds and associates.[3]

During the course of studies of hepatic metabolism as affected by oral feedings of meat and glucose in normal and cirrhotic subjects,[4] it was noted that feedings of meat were frequently accompanied by remarkable rises in HVWP. The purpose of this study is to record our observations on HVWP as affected by the feeding of meat and glucose to normal and cirrhotic subjects, in the hope that some further insight might be gained into the management of the patient with cirrhosis of the liver with portal hypertension.

MATERIALS AND METHODS

The criteria for patient selection have been described elsewhere.[4] Any cirrhotic subject with ascites was studied within 24 hours after abdominal paracentesis so that he or she was as "dry" as possible at the time of study. The technique of Myers and Taylor[5] for catheter wedging of the hepatic vein was followed. When a single lumen venous catheter was wedged in the hepatic vein it was left undisturbed throughout the study. A specially designed double lumen catheter with an opening 3 inches from the distal (tip) lumen was used in a few instances. This latter double lumen catheter allowed for simultaneous measurement of HVWP and free hepatic vein pressure (HVP) without the necessity for moving the catheter from its wedged position.

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When the double lumen catheter was used in the course of the above studies an interesting observation was made which warrants mentioning. If one observes the level of the saline in two water manometers (one connected to the catheter lumen at the wedged position and the other to the catheter lumen open to the free hepatic vein), it will be noted that they move in opposite directions with respiration, e.g., the wedged rising and free falling with inspiration; the reverse with expiration. This has proved to be a useful added simple check on the position of the catheter, since it occurs only when the distal lumen is in the wedged position. The phenomenon is probably related to the inverse pressure alterations between the chest and abdominal cavity with movement of the diaphragm.

In the early part of the study a strain gauge manometer was used for pressure measurements. In most studies, however, a saline manometer was used, the zero level being taken at 5 cm. below the sternal angle. The saline manometer was connected to the catheter through a three-way stopcock. When pressure measurements were to be made, the infusion fluid (isotonic saline containing heparin) used to keep the catheter patent was connected with the saline manometer and allowed to rise to approximately the 500 mm. mark, at which time the manometer and catheter were connected and the saline level in the manometer allowed to equilibrate with the catheter pressure. Recordings were made only when pressure readings remained stable for 60 to 75 seconds.

Following 3 to 5 control measurements taken at intervals of 5 minutes, the fasting, supine, quiet, and comfortable patient ingested either meat or glucose. The glucose feeding consisted of 100 Gm. of glucose in 500 ml. volume; the meat feeding consisted of 250 Gm. of lean meat (about 60 Gm. protein) in 500 ml. volume (lean beef was previously cooked, homogenized, and then brought to volume with water). Pressure measurements were recorded at 5- to 15-minute intervals for 30 to 90 minutes. Using the above procedures, a group of 10 normal and 5 cirrhotic subjects were given the glucose feeding (Table I), and in 9 normal and 11 cirrhotic subjects the protein feeding was given (Table II).

To evaluate the role of alimentation of a nonabsorbable substance, HVWP was measured in one normal and one cirrhotic subject following the ingestion of 8 tablespoonfuls of hydrophilic psyllium mucilloid (Metamucil) in 500 ml. of water. In an additional cirrhotic patient and a normal individual, HVWP was measured after the intravenous administration of 50 Gm. salt-poor albumin. Observations of the effects of the meat feeding in one subject with tricuspid insufficiency with cardiac cirrhosis and congestive heart failure were also made. These 6 subjects comprise a miscellaneous group listed in Table III.

All data are recorded in mm. H_2O. Where proximal (HVP) and distal (HVWP) lumen pressures were taken simultaneously, they are so noted.

RESULTS

A. Control Periods.—The average HVWP in the 20 normal subjects (Tables I and II) and one subject of Table III, was 103 mm. H_2O with a range from 35 to 162 mm. H_2O, and only 4 patients had pressures over 140 mm. H_2O. The average HVWP in the 19 patients with cirrhosis of the liver (Tables I, II, and III) was 302 mm. H_2O with a range from 172 to 458 mm. H_2O, and only 2 cirrhotic subjects had control HVWP below 200 mm. H_2O. In all the normal subjects the free HVP was almost identical with the systemic venous pressure and thus the gradient between HVWP and HVP was uniformly small. The free HVP in 7 cirrhotic subjects ranged from 30 to 111 mm. H_2O with a mean of 72 mm. H_2O. The HVWP-HVP gradient ranged between 153 and 406 mm. H_2O in the cirrhotics, and was thus uniformly high.

B. Glucose Feeding (Table I).—

Normal subjects: In the group of 10 normal subjects glucose ingestion rarely caused a marked increase in HVWP. The greatest increase in HVWP noted in any subject was 34 mm. H_2O, and the average peak increase for the 10 subjects was 17 mm. H_2O.

Cirrhotic subjects: The HVWP rose 50 mm. H_2O in one of the 5 cirrhotic subjects who ingested glucose; in the others the HVWP rose from 5 to 18 mm. H_2O.

TABLE I. EFFECTS OF GLUCOSE FEEDING ON HVWP IN NORMAL AND CIRRHOTIC SUBJECTS

PATIENT NO.	DATA RECORDED IN mm. H_2O PRESSURE		
	CONTROL HVWP	AFTER FEEDING	PRESSURE CHANGE
	A. Normals, Maximum HVWP		
1	60	75	+15
2	35	55	+20
3	114	148	+34
4	81	95	+14
5	87	121	+34
6	108	108	0
7	123 (30)*	144 (30)	+21
8	76	82	+ 6
9	145	160	+15
10	162	182	+20
Mean	100	117	+17
	B. Cirrhotic Subjects		
11	242	260	+18
12	183	195	+12
13	453 (47)	503 (60)	+50
14	384	396	+12
15	435	444	+ 5
Mean	339	360	+21

*Data in parentheses represent simultaneously recorded free HVP.

TABLE II. EFFECTS OF MEAT FEEDING ON HVWP IN NORMAL AND CIRRHOTIC SUBJECTS

PATIENT NO.	CONTROL HVWP	AFTER FEEDING	PRESSURE CHANGE
	A. Normals, Maximum HVWP		
16	108	148	+40
17	76 (44)	116 (66)	+40
18	55	66	+11
19	79	84	+ 5
20	95	141	+46
21	126	162	+36
22	86	151	+65
23	148 (116)	244 (132)	+96
24	162	188	+26
Mean	104	144	+40
	B. Cirrhotic Subjects		
25	370	510	+140
26	172	216	+ 44
27	338	405	+ 67
28	235	266	+ 31
29	364	567	+203
30	285 (71)	337 (80)	+ 52
31	458 (97)	467 (112)	+ 9
32	449 (79)	454 (46)	+ 5
33	205	218	+ 13
34	454	464	+ 10
35	270	325	+ 55
Mean	327	385	+ 58

C. Meat Feeding (Table II).—

Normal subjects: In 9 normal subjects the average peak increase in HVWP after the meat feed was 40 mm. H_2O with a range from 5 to 96 mm. H_2O.

Cirrhotic subjects: In 11 cirrhotic subjects the average peak increase in HVWP after the ingestion of protein was 58 mm. H_2O with a range of from 5 to 203 mm. H_2O. Marked variability in results is apparent. In 5 of these subjects the average peak increase ranged from 31 to 67 mm. H_2O; in 4 of the subjects the average peak increase ranged from 5 to 13 mm. H_2O; in 2 patients the peak increase was 104 and 203 mm. H_2O. In the 3 cirrhotic subjects in whom the pressure was measured in the free hepatic vein simultaneously, the maximal changes were increases of 9 and 15 mm. H_2O, respectively; in one patient the free hepatic vein pressure fell 33 mm. H_2O after the protein feed.

D. Miscellaneous (Table III).—In the two cirrhotic subjects fed nonabsorbable bulk (34 and 35) the peak increase in HVWP was 20 and 22 mm. H_2O, and the peak increase in free HVP was 18 and 2 mm. H_2O. Fifty grams of albumin in 100 ml. of fluid was administered intravenously in 15 minutes to one cirrhotic subject, and this caused the HVWP to increase 16 mm. H_2O with a simultaneous increase in HVP of 21 mm. H_2O; in one normal subject this procedure caused the HVWP to increase 5 mm. H_2O.

TABLE III. MISCELLANEOUS GROUP

SUBJECT	DIAGNOSIS	TYPE FEEDING	CONTROL HVWP	PEAK HVWP	CHANGES
36	Cirrhosis	Metamucil	274 (30)	294 (46)	+20
37	Cirrhosis	Metamucil	223 (70)	245 (72)	+22
38	Normal	I.V. albumin	127	132	+ 5
39	Cirrhosis	I.V. albumin	349 (111)	365 (132)	+16
40	RHD; CHF; T.I.*	Meat	353 (350)	425 (427)	+72

*Rheumatic heart disease; congestive heart failure; relative tricuspid insufficiency and cardiac cirrhosis.

In the patient with rheumatic heart disease (40), the HVWP and free HVP were almost identical in the control periods—353 and 350 mm. H_2O—and both rose—72 and 77 mm. H_2O, respectively—after the meat feeding.

DISCUSSION

The control data in this study are in agreement with the reports of others.[6-8] In the normal subject, the HVWP averages about 100 mm. of water. In the cirrhotic subject the HVWP averaged over 300 mm. of water. There was no overlap in our studies in the HVWP between cirrhotic subjects and normal individuals. Thus it appears, from this and other studies, that a high HVWP is a good criterion for the diagnosis of cirrhosis of the liver. It is generally accepted that the HVWP reflects accurately the sinusoidal pressure, and as such it is an excellent reflection of portal vein pressure. Reynolds and associates[2] and Friedman and Wiener[7] demonstrated the closeness between portal vein pressure and hepatic vein wedge pressure. This increased HVWP of the cirrhotic subjects is undoubtedly due to the morphologic changes produced

in the liver by this disease. Our data also verified the abrupt gradient from occluded to free HVP in the cirrhotic subject. As has been reported by Reynolds and co-workers[2, 3] and Sherlock and associates[8] and others, this observation localized the site of obstruction of blood flow in the cirrhotic liver.

It has been demonstrated that for ascites to develop in the dog, an increase in portal vein pressure is insufficient and that an increase in HVP, brought about by an increase in inferior vena cava pressure above the level of entrance of the hepatic vein will cause ascites.[9] It is known that portal vein occlusion in the human with normal liver function often will not be followed by ascites.[10] However, in some patients with normal liver function, blockage of the portal vein will cause ascites. It is probably quite true that portal vein hypertension is not the sole cause of ascites in the cirrhotic subject. Our data clearly show that there is no increase in free HVP in the cirrhotic subject, and many of our patients did have ascites (see data in parentheses, Tables I and II). Thus, we believe that, though portal venous hypertension per se may not cause ascites, free hepatic vein hypertension is not present and plays no role in the etiology of ascites in patients with cirrhosis of the liver.

Hofbauer studied the effects of feedings on portal vein pressure in the dog and demonstrated that the ingestion of a mixed-type diet in normal dogs caused an increase in portal vein pressure from about 75 mm. H_2O (control) to about 140 mm. H_2O, in most, though not all, animals; in one mongrel dog the ingestion of 300 Gm. of meat caused the portal vein pressure to double.[11] Acute liver damage did not cause an increase in portal vein pressure. In dogs with chronic liver damage caused by carbon tetrachloride, the fasting portal vein pressure was about 30 mm. H_2O above that which had existed before the carbon tetrachloride poisoning. In one dog with chronic liver damage, feeding caused the portal vein pressure to increase from about 135 to 220 mm. H_2O. Feeding also caused a greater absolute and percentage increase in portal vein pressure in those dogs in whom the portal vein had been partially constricted.

In our studies, the ingestion of glucose rarely caused a marked increase in the HVWP in the normal or cirrhotic subject; in only one cirrhotic subject did the HVWP increase 50 mm. H_2O after the ingestion of glucose, and in the remaining subjects the increase was always less than 20 mm. H_2O. The ingestion of meat caused the HVWP to increase by more than 30 mm. H_2O in 7 of 12 cirrhotic subjects; in 2 cirrhotic subjects the increases in pressure were 140 and 203 mm. H_2O. In one normal subject the ingestion of meat caused the HVWP to increase 96 mm. H_2O. Thus, although there was a considerable overlap between the effects of the glucose and meat feedings on the HVWP, and although the increase in the HVWP after meat ingestion was inconstant, it seems significant that it is only with meat ingestion that we did see marked changes in HVWP.

Our data do not provide information as to the cause of the increase in HVWP when it did occur. Intravenous albumin causes a significant rise in

hepatic blood flow,[12] and we have shown that its administration caused but minimal increases in HVWP, and that the ingestion of a moderate amount of nonabsorbable bulk material similarly caused little change in HVWP. If these data are confirmed in a larger group of subjects, it would seem that it is the digestion of meat in the gastrointestinal tract or the liberation of some substance during this digestion, or the secretion of some substance by the gastrointestinal tract itself after the ingestion of meat, that causes the marked increase in HVWP when it does occur. Hoffbauer demonstrated that the administration of epinephrine or histamine caused the portal vein pressure of the dog to increase; histamine probably caused this by its constrictor effect on the hepatic vein.[11] He tentatively concluded that feeding caused an increase in intra-abdominal pressure and then an increase in pressure in the inferior vena cava and portal vein. Our data, with the slight effects on HVWP induced by the ingestion of glucose or inert bulk, and the occasional marked effect brought about by the ingestion of a similar volume of a meat feeding, indicate that the type of material ingested probably also plays an important role in the resulting HVWP.

We have previously demonstrated that a meat feeding frequently causes a significant increase in hepatic blood flow, whereas a glucose feeding usually results in no such increase.[4] Since meat ingestion frequently causes an increase in hepatic blood flow and HVWP, it may well be that an increase in blood flow into the liver, or into that portion of the gastrointestinal tract the venous drainage of which is into the liver, is increased after such a feeding.

Other than by direct measurement of the HVWP before and after a meat feeding, we know of no way by which one would be able to predict the effect of meat ingestion on the HVWP in a specific cirrhotic subject. Since some cirrhotic subjects do manifest a marked increase in HVWP after protein ingestion, and since this is undesirable, it seems wise from this point of view also to limit protein ingestion in the patient with cirrhosis of the liver. We know that protein ingestion may, in some, and again not all, cirrhotic subjects, cause a marked increase in the blood level of ammonia and other nitrogenous substances, and that this may precipitate hepatic coma.[13] We know of at least a few patients in whom bleeding from esophageal varices occurred after hospitalization and after forced high-protein feedings had been instituted. It may well be that this phenomenon was due to an increase in portal vein pressure and was not coincidental.

Thus, from several points of view, it would appear wise to maintain the patient with cirrhosis of the liver in positive nitrogen balance by utilizing proteins with high biologic value, rather than to attempt the forced feeding of a high-protein diet to all cirrhotic subjects.

CONCLUSIONS

1. The HVWP and free HVP were measured in a group of normal and cirrhotic subjects before and after the ingestion of a protein and a glucose feeding.

2. The control HVWP was considerably elevated in all cirrhotic subjects. An increased HVWP may be a criterion for the diagnosis of uncomplicated cirrhosis of the liver.

3. A steep gradient between the HVWP and free HVP was seen only in cirrhotic subjects.

4. The ingestion of glucose was not followed by a significant increase in the HVWP in normal or cirrhotic subjects.

5. The ingestion of a meat feed caused a marked increase in the HVWP of some cirrhotic patients.

6. The administration of intravenous albumin and the ingestion of inert bulky material caused no significant change in the HVWP.

7. The possible clinical significance of these observations is discussed.

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IN VITRO SENSITIVITY OF NORMAL HUMAN LYMPHOCYTES TO X-RAYS AND RADIOMIMETIC AGENTS

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A SLIDE chamber method was recently developed to test the sensitivity of human lymphocytes to reagents.[1] The method required only small quantities of cells and serum and gave quantitative results. In the present study the method was used to measure the cytotoxicity of x-rays and radiomimetic agents on human lymphocytes derived from blood and lymph nodes. These experiments were designed to serve as controls to parallel experiments on the reactions of lymphocytes from patients with chronic lymphocytic leukemia or with lymphosarcoma in leukemic phase.[2] The experiments also were needed to determine to what extent findings on the effects of x-rays on rabbit[3] and rat[4] lymphocytes may be extended to human lymphocytes. Finally this work with the familiar reagent of x-rays helped to explore and develop the use of human cells in the slide chamber method so that other reagents could be used in future studies.

MATERIALS AND METHODS

Description of Patients.—Tests were performed on the lymphocytes from lymph nodes removed for diagnostic purposes from 9 men. The ages of the patients varied from 26 to 70 years. The nodes were obtained from the cervical, axillary, or inguinal regions and were diagnosed in the pathology department of this hospital as chronic lymphadenitis, reactive hyperplasia, and, in 2 nodes, lipomelanotic reticulosis. The clinical diagnoses of the patients were varied and included such conditions as subacute bacterial endocarditis, metastatic carcinoma, exfoliative dermatitis, and duodenal ulcer. The routine hematologic findings were normal except for anemia (lowest hematocrit reading, 29 per cent) and slight leukocytosis (highest count, 13,300 cells per cubic millimeter) in a few patients.

Lymphocytes were also derived from the blood of 28 ambulatory patients between the age of 25 and 72 years. The clinical diagnoses were unusually minor conditions as hemorrhoids and mild acute pharyngitis but also included hypo- and hyperthyroidism and eczematoid dermatitis. Anemia was present in 3 patients with a red cell count of 2,200,000 per cubic millimeter in 1 patient. The white blood cell count varied from 4,000 to 13,300 cells per cubic millimeter usually with 30 to 40 per cent lymphocytes. One patient with rheumatoid arthritis had a white blood cell count of 9,600 with 79 per cent lymphocytes. The sedimentation rate was low in 6 patients (12 to 22 mm. per hour).

The lymphocytes from the lymph nodes and from the blood may be considered as normal, hyperplastic, or inflammatory. These cells should serve as satisfactory controls to the leukemic lymphocytes studied previously.[2]

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Method.—A detailed description of the slide chamber method is given in a previous publication.[1] The normal blood (10 ml.) plus heparin (0.2 ml. of a 0.2 per cent solution) was centrifuged for 4 successive times at 27 g for 5-minute intervals with withdrawal of the turbid plasma after each centrifugation. The cumulated plasma was centrifuged again for 5 minutes at 64 g and the resultant supernatant centrifuged at 110 g. The precipitated cells were washed twice with Earle's solution containing 10 per cent normal serum. The cells were suspended in equal parts of a saline solution (TC 199) and normal human serum of the same ABO type as the cells. This differential centrifugation resulted in a suspension of leukocytes with relatively small numbers of erythrocytes and platelets. Cellular suspensions were also prepared from human lymph nodes by chopping the tissue with fine scissors and filtering through 80-mesh Monel metal wire screen. The suspensions were counted by the method of unstained cell counts and were diluted to give 1,500 viable cells per cubic millimeter.

To prepare the slide chambers, a metal plate, 40 by 40 by 0.85 mm. with a central hole 26 mm. in diameter, was placed in the center of a large cover glass, 50 by 75 mm. A thin rectangle about 12 by 15 mm. was drawn with petroleum jelly on the cover glass inside the hole of the metal plate. A drop of suspension, 0.2 ml., was placed inside the petroleum jelly ring and another cover glass, 43 by 50 mm., was lowered on the drop. The preparation was then sealed with a beeswax-paraffin mixture.

The cellular suspension in the slide chamber was irradiated with a 100 kv. x-ray machine and the same factors were used as previously.[2] The dosage of 1,000 to 4,000 r was administered at the rate of 670 r per minute, 5 Ma., 0.5 mm. equivalent aluminum filter, distance of 10.5 cm. and half-layer value of 1.3 mm. aluminum; 100 r, at the rate of 67 r per minute and distance of 31.5 mm.; 10 r or less, at the rate of 7.5 r per minute, 31.5 distance, 0.25 mm. copper and 0.5 mm. equivalent aluminum filter, and HLV of 0.94 mm. aluminum.

The radiomimetic agents were made up fresh in distilled water and added in suitable dilutions to the suspension immediately before the preparation of the slide chambers. The nitrogen mustard was NH2, methyl-bis(β-chloro-ethyl)amine hydrochloride.* The triethylene melamine was kindly contributed by Dr. J. M. Ruegsegger.†

The slide chambers were incubated at 37° C. The cells settled rapidly in a single layer on the lower cover slip and were examined with an inverted phase microscope. Counts were made daily of the viable lymphocytes in a strip 0.215 mm. wide and 10 mm. in length. The criteria used for recognizing the viable, and morphologically intact lymphocyte included (1) a clearly visible nucleus with dark chromatin and nucleolar masses imbedded in a light-gray nucleoplasm; (2) clearly visible, but not prominent, cell and nuclear outlines; and, (3) in some cells, a broad, anterior pseudopod and a posterior tail with fine granules and mitochondria.

RESULTS

Observations on Untreated Lymphocytes.—Suspensions prepared from blood lymphocytes in 50 per cent human serum were studied by phase microscopy and time-lapse cinemicrography. In a fresh suspension, the lymphocytes were round with a small amount of cytoplasm. The nucleus was finely granular with a few small, indistinct chromatin masses distributed irregularly in a light-gray nucleoplasm. A few of the chromatin masses were attached to the nuclear wall but margination of the chromatin was not conspicuous. After one or more days of incubation in the slide chambers, most of the blood lymphocytes were ameboid in form with broad hyaline anterior pseudopods and short narrow posterior tails which contained a few granules and mitochondria. In time-lapse cinemicrography, these cells were seen to be active, lashing about aimlessly or moving in one direction at a slower rate than granulocytes.

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Suspensions from the lymph nodes were variable in cytology. In most of the suspensions the predominating cell was similar to the blood lymphocyte as indicated by coarse, irregularly distributed chromatin masses and by their ameboid form after one or two days of incubation. In a few suspensions many of the lymphocytes were smaller with cart wheel nuclei and these cells usually remained round and nonmotile. A few cells were lymphoblasts with hyaline nuclei, prominent nucleoli, and no definite chromatin masses. The lymphoblasts were usually irregular in shape and were motile shortly after incubation. Other types of cells were seen in some suspensions. For example, a suspension from a lymph node of one patient contained many reticular cells with radiating fibrillary processes. The findings on this and a few other unusual suspensions are not included in this study.

TABLE I. A SUMMARY OF THE FINDINGS ON THE SENSITIVITY OF HUMAN LYMPHOCYTES TO X-RAYS (1,000 r), NITROGEN MUSTARD (3 μg OF HN2 PER ML.), AND TRIETHYLENE MELAMINE (0.3 μg OF TEM PER ML.)

	ARITHMETIC MEAN		RANGE	
	LYMPH NODES	BLOOD	LYMPH NODES	BLOOD
Ten per cent survival time of untreated cells (days)	7.73	9.23	5.8 to 10.3 (7)	6.2 to 14.8 (26)
Sensitivity; 10 per cent survival time in days after:				
X-rays (1,000 r)	1.48	1.71	1.28 to 1.87 (6)	1.08 to 2.24 (19)
HN2 (3 μg/ml.)	2.16	2.04	1.67 to 2.48 (3)	1.17 to 2.93 (7)
TEM (0.3 μg/ml.)	1.56 (1)	2.21	—	1.87 to 2.44 (4)
Sensitivity-dose producing a 10 per cent survival time of 3 days:				
X-rays (roentgens)	62	111		
HN2 (μg/ml.)	2.31	2.05		
TEM (μg/ml.)	—	0.07		
Slope of dose-effect curve, after:				
X-rays	1.55 (5)	1.78 (6)		
HN2	2.56 (3)	3.04 (3)		
TEM	—	1.20 (3)		

Numbers in parentheses indicate numbers of patients.

In untreated suspensions from 8 lymph nodes, 44 to 88 per cent of the lymphocytes were still viable in the slide chambers after 4 days of incubation at 37° C. The corresponding figures for blood lymphocytes of 27 patients were 30 to 88 per cent. The median percentages of lymphocytes viable after 4 days of incubation was 55 per cent for the lymph node and 65 per cent for the blood lymphocytes. The average 10 per cent survival time was 7.7 days for the cells of the lymph nodes and 9.2 days for the blood lymphocytes (Table I). To summarize, normal human lymphocytes had a 50 per cent survival time of more than 4 days, and 10 per cent of the cells survived about 8 days. Furthermore, the lymphocytes of the blood showed somewhat better survival than those from the lymph nodes under the present conditions of investigation.

A few incidental observations were made on other types of cells in normal blood. The neutrophils survived only 2 to 4 days while the eosinophils and monocytes survived many days. After 6 or 8 days, a few suspensions derived from normal blood developed large macrophages with large hyaline nuclei, prominent nucleoli, and many large granules. Incompatibility of the cells and serum due to different ABO blood groups caused the granulocytes to phagocytize and hemolyze the red blood cells with the production of multiple large cytoplasmic vacuoles.

X-Rays.—The morphologic changes in human lymphocytes irradiated with x-rays were studied both by time-lapse cinemicrography and by daily observations of the slides. After irradiation with 4,000 r, many lymphocytes from lymph nodes and from blood developed, in a few hours, nuclei which were bi- or trilobed or had other irregularities in shape. In time-lapse cinemicrography the cells had an oscillatory movement which became motionless a few hours after irradiation. The loss of oscillatory movements was considered to be an indication of the death of the cell. This death developed both in lymphocytes with normal morphology and in those with lobated nuclei. The killed cells were usually round and the nucleus had dark chromatin masses with very light nucleoplasm. The dead lymphocyte underwent slow postmortem changes and developed a shrunken round nucleus, blurred chromatin masses, a heavy nuclear wall, clear edematous cytoplasm, and a thin prominent cell wall. Ultimately the nucleus disappeared leaving a small, shrunken granular cell. The changes in the human lymphocyte after 4,000 r were similar to those observed in rabbit thymocytes treated with 4,000 or 10,000 r and described previously as death by delayed fixation.[5]

After treatment with 1,000 r or less, the human lymphocytes developed a large intranuclear vacuole surrounded by a dark chromatin ring. Most, but not all, degenerating cells underwent rapid changes in shape with the formation of lobulations. The chromatin ring persisted for a short time, then ruptured and contracted to give a structureless dark pyknotic nucleus. This process of death by intranuclear vacuolization and lobulation was similar to that observed in rabbit thymocytes after 1,000 r.[3]

The survival curves of the irradiated and nonirradiated lymphocytes of the lymph node from Patient H60 is shown in Fig. 1 on semilogarithmic paper. The curves are not linear indicating that death of these cells cannot be considered a mononuclear reaction. The 10 per cent survival time was 1.3 days after 1,000 r and 5.4 days after 2 r. After 5.7 days' incubation, the cells in the control suspension showed 38 per cent survival while the cells treated with 2 r had a survival of only 0.8 per cent.

Several experiments were done to determine the minimum dosage that produced an effect on the lymphocytes from lymph nodes of 5 and from the blood of 2 patients (Table II). In 3 experiments, 2 r produced a small (3, 9, and 19+ per cent) but consistent decrease in the 10 per cent survival time of the lymphocytes from 3 lymph nodes. A dosage of 3 to 5 r produced greater

(14 to 39+ per cent) decreases in the 10 per cent survival times. It may be concluded that human lymphocytes are sensitive to dosages of x-rays as small as 2 to 5 r.

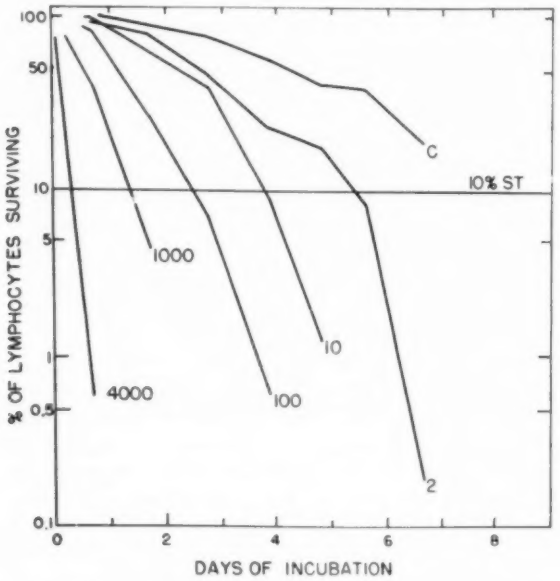

Fig. 1.—Survival curves showing the effect of 2 to 4,000 r of x-rays on the survival of lymphocytes from the lymph node of Subject H60. The node was obtained from the axilla of a 58-year-old white man with the clinical diagnosis of duodenal ulcer. The histologic diagnosis on the node was chronic lymphadenitis.

TABLE II. THE EFFECT OF SMALL DOSAGES OF X-RAYS ON THE 10 PER CENT SURVIVAL TIMES OF HUMAN LYMPHOCYTES FROM LYMPH NODES OF 5 PATIENTS AND FROM THE BLOOD OF 2 PATIENTS

SOURCE	10 PER CENT SURVIVAL TIME IN DAYS AND IN PERCENTAGE OF CONTROL (DOSAGE OF X-RAYS)			
	NONE	2 R	5 R	10 R
Lymph nodes				
H60	6.7+	5.40 (<81 %)	4.08 (<61 %)	3.78 (<56 %)
H70	5.8	5.30 (91%)	4.63 (80%)	3.60 (62%)
H507	8.3	—	—	5.0 (60%)
H557	7.3	—	—	4.12 (56%)
H608	6.8	6.59 (97%)	—	4.08 (60%)
Blood				
H604	8.4	—	7.25* (86%)	5.35 (64%)
H624	6.2	—	4.46† (72%)	3.06 (49%)

*Three roentgens.
†Four roentgens.

The effect of varying amounts of x-rays is shown in the dose-effect curve of Fig. 2 where dosage is expressed in roentgens on a logarithmic scale and the effect as the 10 per cent survival time in days. The data obtained on the

cells from 5 patients can be represented by a single linear curve. A regression equation for the data was calculated to be: $y = 5.78 - 1.55 \log x$ where x is the dosage of x-rays and y is the 10 per cent survival time. In this equation the slope of the line is expressed by the coefficient of log x and is, disregarding the negative sign, 1.55 days per log dose. It should be noted that the slope of the line is an important statistic as it may be considered a measure of the variability of the sensitivity of the individual lymphocytes.

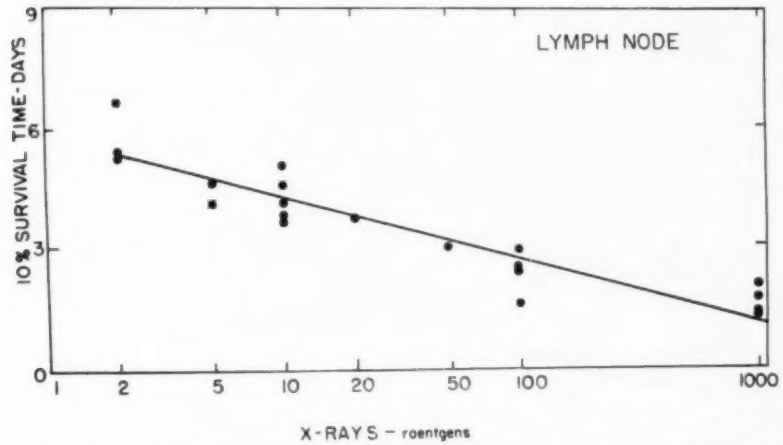

Fig. 2.—A dose-effect curve showing the effect of 2 to 1,000 r of x-rays on the 10 per cent survival time of lymphocytes derived from the lymph nodes of 5 patients. The linear curve in the graph represents the calculated regression line for the equation: $y = 5.78 - 1.55 \log x$.

The regression equation represents the radiosensitivity of the human lymphocytes of lymph nodes. It is, however, desirable to have a single index of radiosensitivity. An index can be set up by taking a single point in the curve using either an arbitrary time interval or an arbitrary dose as standard. The dosages that produced a 10 per cent survival time of 3 days is, according to the regression equation, 62 r, and this figure can be used as an index of the radiosensitivity. A more convenient and direct method of measuring sensitivity is to determine the effect of a given dose of x-rays, such as 1,000 r. The 10 per cent ST-1,000 r was directly measured for 6 lymph nodes and was found to vary from 1.28 to 1.87 days with an average of 1.48 days (Table I).

The effect of x-rays on lymphocytes from the blood of nonleukemic individuals is presented in Fig. 3. The data for the cells of 6 individuals can be represented by a single linear dose-effect curve with this regression equation: $y = 6.65 - 1.78 \log x$. The slope of the curve is 1.78 which is not definitely different from that (1.55) for lymph nodes in Fig. 2. It has been seen previously that 62 r of x-rays produced a 10 per cent survival time of 3 days in lymphocytes from lymph nodes. The comparable figure for lymphocytes of blood is 111 r. The lymphocytes from human blood appeared to be less sensitive to x-rays than those from human lymph nodes.

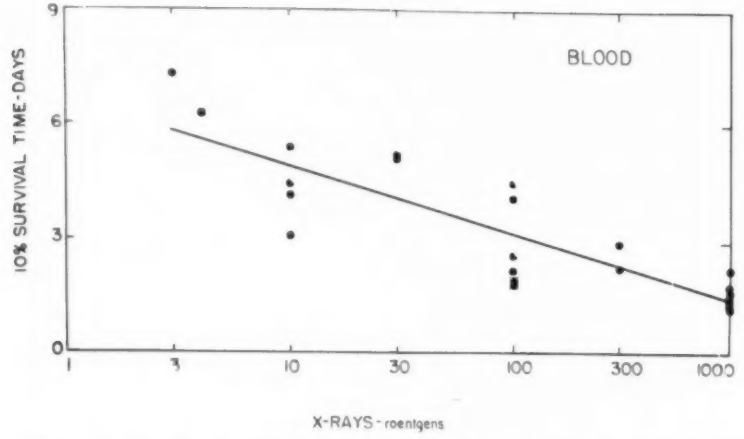

Fig. 3.—A dose-effect curve showing the effect of 3 to 1,000 r of x-rays on the 10 per cent survival times of lymphocytes derived from the blood of 6 patients. The regression line represents the equation: $y = 6.65 - 1.78 \log x$.

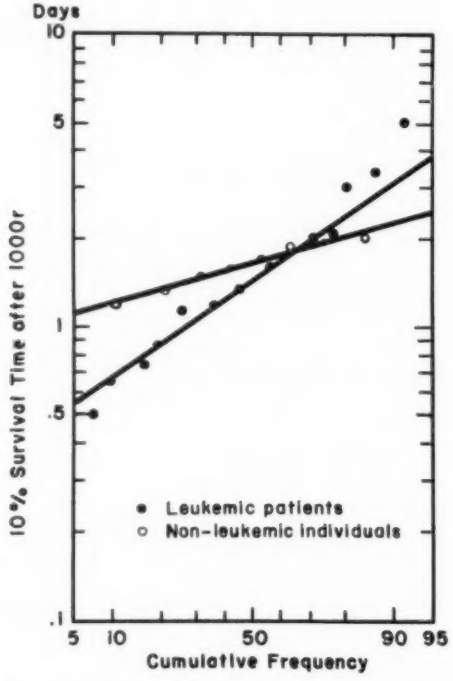

Fig. 4.—Probit curves showing the frequency distributions of the 10 per cent survival time of lymphocytes irradiated with 1,000 r. The cells were derived from the blood of 19 normal individuals and from 31 patients with chronic lymphocytic leukemia or lymphosarcoma in leukemic phase.

A group of 19 normal individuals were studied to determine the variability of the measure of radiosensitivity of their blood lymphocytes to 1,000 r, and the data are summarized in Table I. The 10 per cent survival time after 1,000 r varied from 1.08 to 2.24 days with an arithmetic mean of 1.71 days, a standard deviation of 0.32 days, and a coefficient of variation of 19 per cent. It should be noted that the observed variability included both the experimental error of measurement and the variability of the reactions of lymphocytes from patients with a variety of diseases.

Fig. 4 presents a cumulative frequency curve of 10 per cent ST-1,000 r for blood lymphocytes of 19 nonleukemic individuals. The ordinates are the logarithms of the 10 per cent ST-1,000 r and the abscissas, the cumulative frequency in probability units. It is seen that the curve is a straight line with a median of 1.7 days. The figure also shows the previously published cumulative curve for 31 patients with chronic lymphocytic leukemia or lymphosarcoma in leukemic phase. The difference in slopes of the 2 curves is a graphic representation that the patients with leukemia are more variable in respect to radiosensitivity of their blood lymphocytes than nonleukemic individuals.

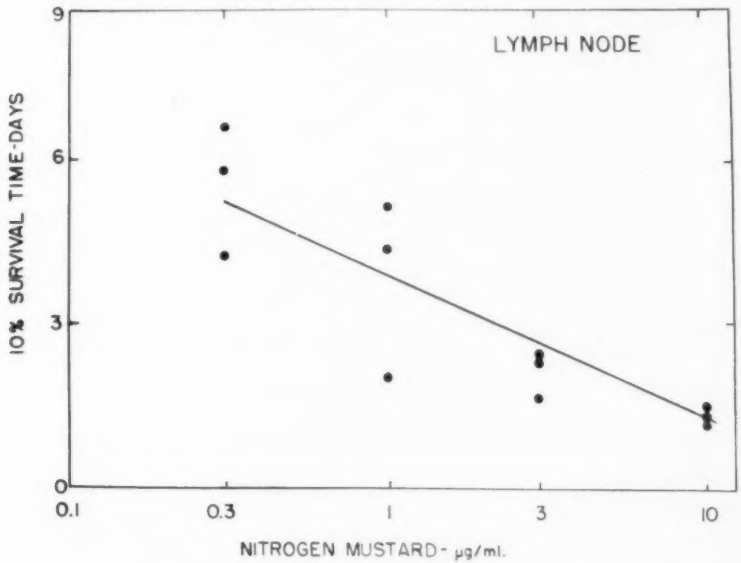

Fig. 5.—A dose-effect curve showing the effect of 0.3 to 10 µg of nitrogen mustard per milliliter of suspension on the 10 per cent survival times of lymphocytes derived from the lymph nodes of 3 patients. The regression line represents the equation: $y = 3.93 - 2.56 \log x$.

Radiomimetic Agents.—A dose-effect curve was obtained for the cytotoxic action of nitrogen mustard on the lymphocytes of lymph nodes of 3 patients (Fig. 5). The regression equation is: $y = 3.93 - 2.56 \log x$. The cor-

responding dose-effect curves for blood lymphocytes are given in Fig. 6. The statistical data are summarized in Table I. Treatment of lymphocytes with 3 μg per milliliter of nitrogen mustard reduced the average 10 per cent survival time to 2.16 days for lymph node lymphocytes and to 2.04 days for blood lymphocytes. The slopes of the dose-effect curves were approximately 2.56 days per log dose for the lymph nodes between dosages 0.3 and 10 μg per milliliter and 3.04 for blood lymphocytes between dosages 1.0 to 10.0. These slopes are greater than those for the effect of x-rays on lymphocytes of lymph node and blood.

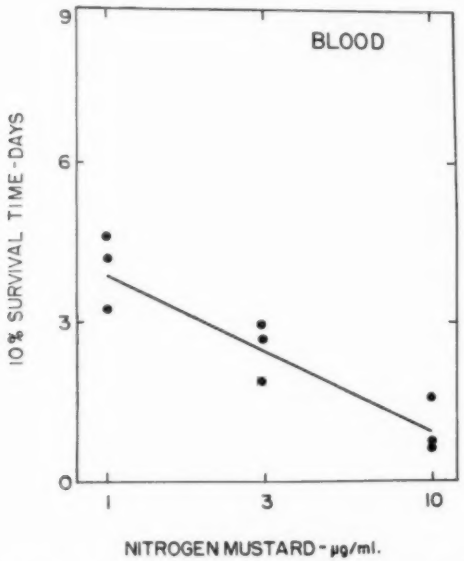

Fig. 6.—A dose-effect curve showing the effect of 1 to 10 μg per milliliter of nitrogen mustard on the 10 per cent survival times of lymphocytes derived from the blood of 3 patients. The regression line represents the equation: $y = 3.94 - 3.04 \log x$.

The effect of triethylene melamine on the lymphocytes of blood from 3 individuals is shown in Fig. 7. The slopes of the dose-effect curves are relatively low, 1.20 days per log dose. A dosage of 0.3 μg per milliliter resulted in a 10 per cent survival time of 2.21 days. It is seen in Table I that, according to the cytotoxic effect on human blood lymphocytes, a dosage of 0.3 μg per milliliter of triethylene melamine was approximately equivalent to 3.0 μg per milliliter of nitrogen mustard and to 1,000 r of x-rays.

Incidental cytologic observations were made on the effects of the chemical agents on lymphocytes. Large doses of nitrogen mustard (30 μg per milliliter) or of triethylene melamine (3 μg per milliliter) caused the formation of bi- and trilobed nuclei, cessation of oscillatory movements, and death with temporary preservation of nuclear structures followed by slow post-mortem

changes. Lower dosages of the two agents (3 and 0.3 μg per milliliter, respectively) caused intranuclear vacuolization with or without lobulation and death with the formation of a dark structureless pyknotic nucleus. The radiomimetic agents produced the same two types of death as x-rays, namely, delayed fixation and intranuclear vacuolization.

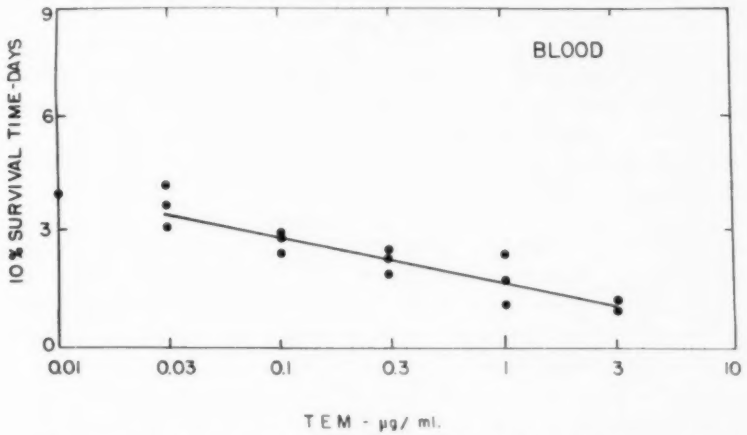

Fig. 7.—A dose-effect curve showing the effect of 0.01 to 3 μg per milliliter of triethylene melamine on the 10 per cent survival time of lymphocytes derived from the blood of 3 patients. The regression line represents the equation: $y = 1.62 - 1.20 \log x$.

DISCUSSION

This paper presents data on the sensitivity of human lymphocytes to x-rays and radiomimetic agents. In this initial survey only a few individuals were tested and the statistics represent only a first approximation. The findings depend, of course, on the experimental conditions used and further studies are needed to determine the effect of variation of the conditions on the indices of sensitivity.

This study shows the feasibility of the slide chamber method to provide quantitative and qualitative data. The method measures such quantities as, the survival time of cells in vitro, the cytotoxicity of a reagent, the rapidity of its action, and the sensitivity of different types of cells to a reagent. The method also provides almost ideal conditions for the microscopic and cinemicrographic study of cells for prolonged periods of time.

The present findings may be compared to those obtained previously on the radiosensitivity of human blood cells by the method of unstained cell counts.[6] It should be first noted that the present and previous experimental conditions were quite different. In the present method, the suspension contained about 1.5 cells per micromilliliter, the cells were irradiated and incubated in a chamber containing only a small amount of air (equal to 1.2 volumes of liquid); the cells were quiescent and resting on glass during the incubation, and the morphologic characteristics of the lymphocytes were used as criteria

of viability. In the unstained cell count method, the suspension contained about 100 cells per micromilliliter; the cells were irradiated and incubated in a test tube containing a considerable amount of air (20 times the volume of suspension). During incubation the cells were shaken continuously, and the viability was determined by the capacity of the cells to resist staining with eosin. The comparison of the two methods is limited by the fact that in the chamber method only the lymphocytes were counted while all the nucleated cells were counted by the unstained cell method. According to the chamber method, human blood lymphocytes had a 10 per cent survival time of 9.2 days which was reduced to 1.7 days after irradiation with 1,000 r. With the unstained cell method, the blood cells had a 10 per cent survival of 5.5 days for untreated cells and 2.6 days for irradiated cells. In spite of the differences in the experimental conditions, the 2 methods gave comparable results.

One finding in the present work seemed surprizing and is worthy of note. The human lymphocytes were found to give an appreciable reaction to irradiation with as small a dose as 5 r or less. Few biologic reactions are sensitive to this dose. In contrast, the lymphocytes from the rabbit thymus required about 50 r to produce a perceptible decrease in the survival time according to the method of unstained cell counts.[6]

In comparison to the lymph node lymphocyte, the blood lymphocyte required about 2 times the dosage to produce the same effect in 3 days. Trowell[4] found that the blood lymphocytes of the normal rat required 6 times the dosage as the lymphocyte from the lymph node to produce the same amount of damage in 5 hours. In both rat and man, the lymphocyte of the blood seemed less radiosensitive than those from the lymph node. It is not known, however, whether the observed lower radiosensitivity of the human blood lymphocyte is due to (1) an innate difference in the two types of lymphocytes, (2) differences in experimental conditions such as the greater number of granulocytes associated with the blood lymphocytes, (3) differences in the underlying conditions of the patients who provided the blood and lymph node specimens, or (4) the greater survival time (9.23 days) of the untreated blood lymphocytes compared to that (7.73 days) for lymph node lymphocytes.

SUMMARY

Human lymphocytes from blood and lymph nodes survived satisfactorily in slide chambers with 10 per cent of the cells usually surviving 8 or 9 days. The 10 per cent survival time was reduced to about 2 days by 1,000 r of x-rays, 3.0 μg per milliliter of nitrogen mustard or 0.3 μg per milliliter of triethylene melamine. The slope of the dose-effect curves was greater for nitrogen mustard than for x-rays or triethylene melamine. The lymphocytes of the lymph nodes appeared to be twice as sensitive to x-rays as those of the blood. A dosage of 2 to 5 r sufficed to produce a perceptible toxic effect on the human lymphocytes.

The human lymphocyte, like the rabbit thymocyte, showed two distinct morphologic reactions to x-rays. The lymphocyte died by means of delayed

fixation after treatment with 4,000 r, and by intranuclear vacuolization, lobulation, and pyknosis after 1,000 r. The two radiomimetic agents tested produced the same two types of death depending upon the dosage of the reagents.

The biopsies of lymph nodes were made available through the kind cooperation of Dr. William S. Walsh. My thanks are also extended to June Zerwekh, Melchior Carino, and Burnell Coleman for technical assistance.

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THE USE OF DIISOPROPYLFLUOROPHOSPHATE^{32} FOR THE DETERMINATION OF IN VIVO RED CELL SURVIVAL AND PLASMA CHOLINESTERASE TURNOVER RATES

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DIISOPROPYLFLUOROPHOSPHATE (DFP), one of the alkyl fluorophosphates, is a potent inhibitor of cholinesterase activity in vitro and in vivo. This inhibition is irreversible and will occur in vivo after the oral or parenteral administration of the drug. Serum and erythrocyte cholinesterases are extremely sensitive to the action of DFP, and after it is given their levels will be reduced significantly before peripheral effects are noted.

In 1947, Grob and associates[1] administered DFP to human beings and found that the rate of regeneration of cholinesterase activity in erythrocytes was 1.2 per cent per day. Jandorf and McNamara[2] used radioactive DFP^{32} in rabbits and found that the elimination of P^{32} activity from their erythrocytes paralleled the reappearance of cholinesterase in them. Cohen and Warringa[3] studied red cell and plasma radioactivity after the intramuscular injection of DFP^{32} into 2 patients. In these subjects red cell radioactivity decreased to zero in about 120 days.

The present study was undertaken to evaluate DFP^{32} as an erythrocyte tagging agent with the following points in mind:

1. Red cell and plasma uptake of DFP^{32} after in vivo and in vitro tagging.
2. In vivo survival of erythrocytes labeled with DFP^{32} in vivo and in vitro.
3. Absorption of P^{32} from the gastrointestinal tract after oral administration of DFP^{32}-labeled blood. This is important if DFP^{32}-tagged red cells are to be used for quantitation of blood loss in patients with digestive tract hemorrhage.

METHODS

Diisopropylfluorophosphate^{32}.—DFP^{32} was suspended either in sterile peanut oil† or in propylene glycol.‡

Subjects.—All subjects were adult male hospital personnel in good health. Their hemoglobin and hematocrit values were normal throughout the period of the experiment.

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†Kindly supplied by Dr. J. A. Cohen of the Medical Biological Laboratory of the National Defense Council, Rijesevska, Netherlands.

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Preparation of Samples.—Seven milliliters of blood was drawn into heparinized nylon syringes and placed in silicone-coated centrifuge tubes in an ice-water bath. The blood was centrifuged at 100 g at 5° C. for 15 minutes. The platelet-rich plasma was removed with a silicone-coated pipette and centrifuged at 2,000 g for 20 minutes to remove the platelets. Two milliliters of plasma was pipetted into a planchet for the determination of radioactivity. The red cells were washed 3 times with cold saline (100 g for 15 min.) and resuspended in saline. A hemoglobin determination was done on the cell suspension. Two milliliters of this suspension was pipetted into a planchet and dried at 37° C. for 24 hours before assay in the gas flow counter. When the scintillation counter was used, 5 ml. liquid aliquots were counted in cellulose nitrate test tubes.

Radioactive Counting.—Most radioactive counting was done using the methane gas in a flow window counter* operated in the proportional region. The background was 30 c.p.m. and the efficiency 34 per cent for aqueous P^{32} and 14.2 per cent for P^{32} mixed with the washed erythrocytes from 2 ml. of normal whole blood. In later experiments a beta well scintillation counter† was used. The background was 50 c.p.m. and the efficiency for P^{32} mixed with the washed erythrocytes from 5 ml. of blood, 6 per cent.

Sufficient counts were taken on all samples to give a statistical counting error of less than 3 per cent (1 S.D.),[4] except for samples obtained 90 days after injection of DFP^{32} where the counting error was 5 per cent, and for those obtained after 100 days, 10 per cent.

Total counts injected in the propylene glycol DFP^{32} experiments were obtained by counting a known volume of the injected DFP^{32} in a suspension of erythrocytes of the same concentration as erythrocyte suspensions used during the survival studies.

In the experiments with DFP^{32} in peanut oil, total counts injected were calculated from the counting efficiency for P^{32} erythrocytes and the number of microcuries injected.

The per cent uptake of DFP^{32} by the circulating red cells equaled:

$$\frac{\text{(c.p.m./Gm. hemoglobin at zero time) (total circulating hemoglobin)}}{\text{total counts injected}} \times 100.$$

Measurement of Hemoglobin.—Determinations of hemoglobin were done in duplicate with a Klett-Summerson colorimeter. The cyanmethemoglobin method of Drabkin[5] was used.‡

DFP^{32} Labeling of Erythrocytes In Vitro.—DFP^{32} was added to the plasma layer of centrifuged A.C.D. blood which had been collected in plastic bags.§ To determine red cell uptake of DFP^{32}, aliquots were removed at intervals and washed 3 times in cold saline prior to measuring the P^{32} content of erythrocytes. An unwashed aliquot of the whole blood-DFP^{32} mixture was used as a standard.

DFP^{32} Content of Stools.—One part of stool mixed with 2 to 5 volumes of water was homogenized in a blender. Weighed aliquots were counted and compared to standards made from an aliquot of the ingested blood which had been mixed with homogenized normal stool.

Sodium Chromate^{51} Survival Studies.—Sterile sodium chromate^{51}‖ was added to A.C.D. blood¶ at room temperature for Cr^{51}-labeling of erythrocytes.[6] The concentration was 1.7 μg of chromium per milliliter of packed erythrocytes. One hour later a weighed aliquot of the mixture was injected into the recipient and at the same time a second aliquot was

*Model 210, Packard Instrument Co., Chicago, Ill.

†Baird Atomic Instrument Co., Cambridge, Mass.

‡Hemoglobin standard supplied by the National Academy of Sciences, National Research Council, through the kindness of Dr. William Crosby.

§Fenwal Corporation, Ashland, Mass. (500 ml. whole blood plus 75 ml. U.S.P. and N.I.T. "Formula A" ACD solution).

‖Abbott Laboratories, North Chicago, Ill.

¶Two and five-tenths milliliters of NIH Formula A acid citrate dextrose plus 10 to 15 ml. of whole blood.

washed 3 times in saline for use as a standard. Seven milliliter samples were taken from the recipient at 10 minutes, 24 hours, and at intervals for 52 days. These were washed 3 times in saline and counted in a well-type scintillation detector. No correction for elution was applied.[6]

Calculation of Red Cell Mass and Plasma Volume.—The total red cell volume was estimated as 29 ml. per kilogram of body weight times the subject's hematocrit value over 47.[7] Total body hemoglobin was obtained by multiplying red cell volume by 0.33. The plasma volume was calculated from the estimated red cell volume using the following formula:

$$\text{Plasma volume} = \frac{(\text{red cell volume})\ (100 - 0.9 \times \text{hematocrit})}{0.9 \times \text{hematocrit}}$$

where the plasma volume and red cell volume are in milliliters and the hematocrit in volumes per cent.

RESULTS

Volatility of DFP^{32}.—DFP^{32} volatilizes from peanut oil in an exponential fashion with a half-time of 13.9 ± 4.0 minutes (range 10.5 to 18.6). In one experiment DFP^{32} volatilized from propylene glycol exponentially with a half-time of 85 minutes.

In Vivo Uptake of DFP^{32} by Erythrocytes and Plasma.—Each subject received 0.9 mg. of DFP^{32} in peanut oil, or from 0.5 to 0.7 mg. of DFP^{32} in propylene glycol, by deep intramuscular injection. For each subject a graph was drawn using the number of counts per minute per gram of circulating hemoglobin as the vertical axis and time in days as the horizontal axis. From this graph the number of counts per minute per gram of hemoglobin at zero time was obtained by using the method of least squares to extrapolate the line to the vertical axis.[8]

$$1.\ Y = a + bx$$
$$2.\ \Sigma Y = na + b\Sigma x$$
$$3.\ \Sigma XY = a\Sigma x + b\Sigma x^2$$
$$4.\ \sigma_E = \frac{\Sigma y^2 - (a\Sigma y + b\Sigma xy)}{n}$$

where x = counts per minute per gram of circulating hemoglobin, y = time in days, n = the number of observations, and σ_E = the standard error of prediction.

With DFP^{32} in peanut oil, 38.5 ± 2.7 per cent (range 33.6 to 44.2 per cent) of the injected radioactivity was taken up by erythrocytes. When DFP^{32} in propylene glycol was used, 25.1 ± 3.4 per cent (range 21.1 to 29.0 per cent) was taken up by them.

To obtain the amount of DFP^{32} in the plasma at zero time, the initial steep portion of the plasma radioactivity curve was extrapolated to the vertical axis in Subjects 1, 2, 5, 8, and 16. After injection of DFP^{32} in peanut oil, 8.9 ± 2.9 per cent (range 6.2 to 12.1 per cent) of the radioactivity appeared in the plasma.

Red Cell Survival After Tagging In Vivo With DFP^{32}.—The red cell radioactivity of 8 normal subjects decreased in linear fashion at a rate of 0.80 ± 0.07 per cent per day (Fig. 1) with the extinction point at 124.6 ± 11.0 days.

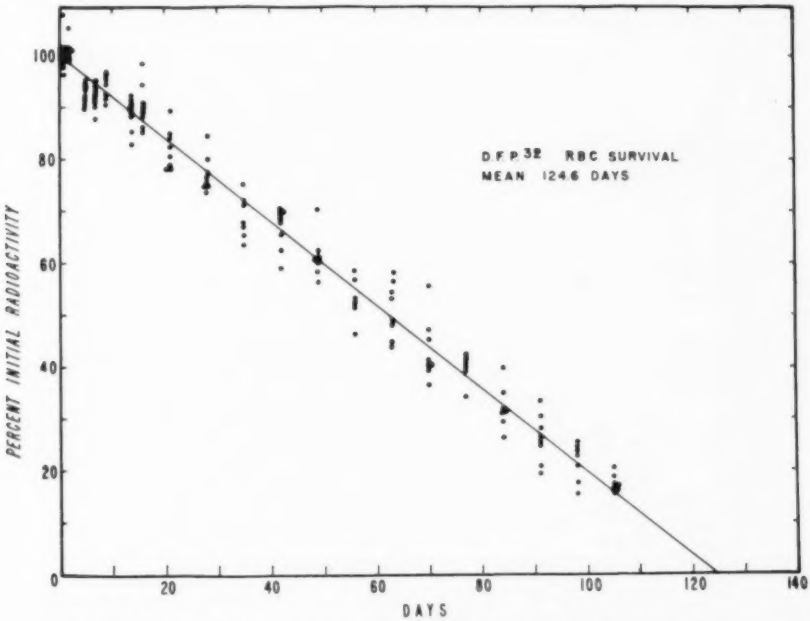

Fig. 1.—For each of 8 subjects a straight line was drawn by the method of least squares using counts per minute per gram of hemoglobin as ordinate and time as abscissa. The value for the point at which this line crossed the ordinate (c.p.m. per gram of hemoglobin at zero time) was taken as 100 per cent. In the above figure all data for each subject were expressed as per cent of the R.B.C. radioactivity (at 0 time).

Because of a rapid initial fall of radioactivity in the red cells of the first 8 volunteers studied (Fig. 1), 4 additional subjects were given intramuscular injections of from 0.5 to 0.7 mg. of DFP^{32} in propylene glycol. In these, 8 observations were made on each subject during the first 10 days (Fig. 2). During this period the average slope was equivalent to an erythrocyte lifespan of 52 ± 7 (range 44 to 59) days. The rate of fall was greatest during the first 5 days. Analysis of the data from the fifth to the thirty-second days showed a mean red cell lifespan of 117 ± 36 days.

Plasma Activity After Labeling In Vivo.—The curves of radioactivity in plasma after in vivo tagging are shown in Fig. 3. The initial rapid decline of the plasma radioactivity is considered to be due to mixing of labeled protein with extravascular protein. The slower exponential components with a half-time of 16.6 ± 5.6 days probably represent the turnover of plasma cholinesterase and the elution, if any, of P^{32} from it.

Labeling of Erythrocytes In Vitro by DFP^{32}.—In 2 experiments DFP^{32} in propylene glycol was added to the plasma layer of centrifuged A.C.D. blood

(2 μg DFP^{32} per milliliter A.C.D. blood), and in 2 experiments DFP^{32} in peanut oil (3 μg and 9 μg DFP^{32} per milliliter A.C.D. blood) was used. The per cent of added radioactivity contained in thrice washed erythrocytes at various intervals of time is shown in Fig. 4.

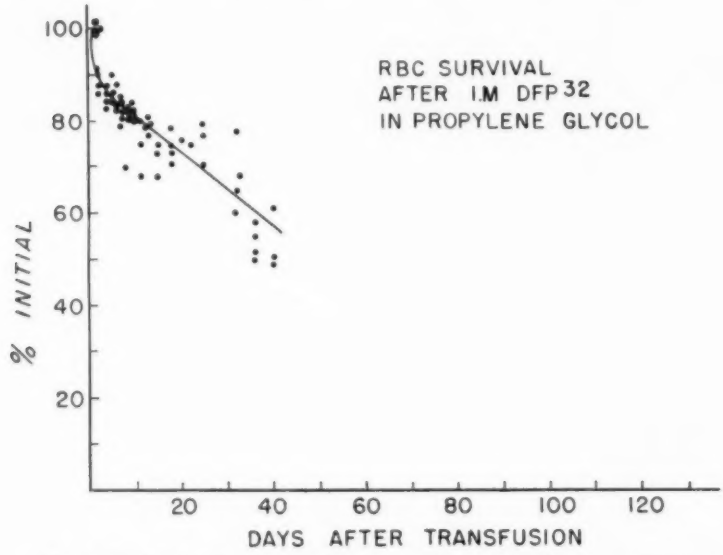

Fig. 2.—Red cell P^{32} activity after an intramuscular injection of DFP^{32} in propylene glycol. All points for each subject are expressed as a percentage of the c.p.m. per gram of hemoglobin the first day after injection of DFP^{32}.

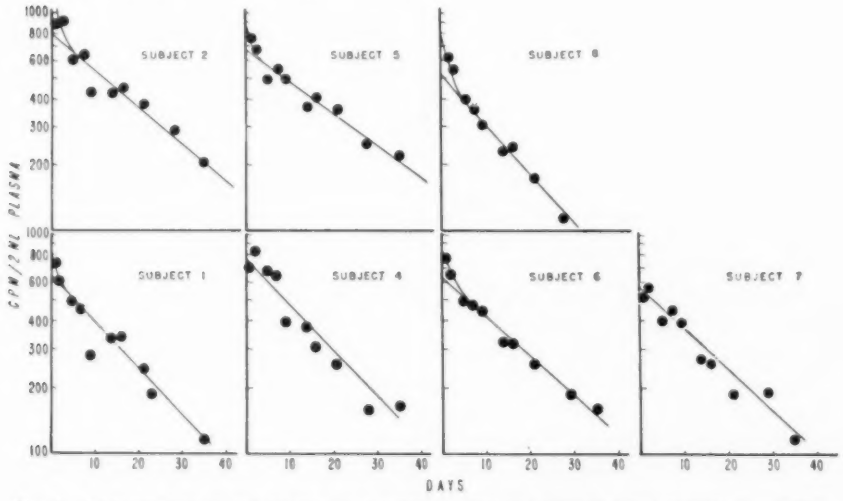

Fig. 3.—Radioactivity in the plasma of 8 subjects after the intramuscular injection of DPF^{32}. Semilogarithmic plot. The lines were drawn by inspection.

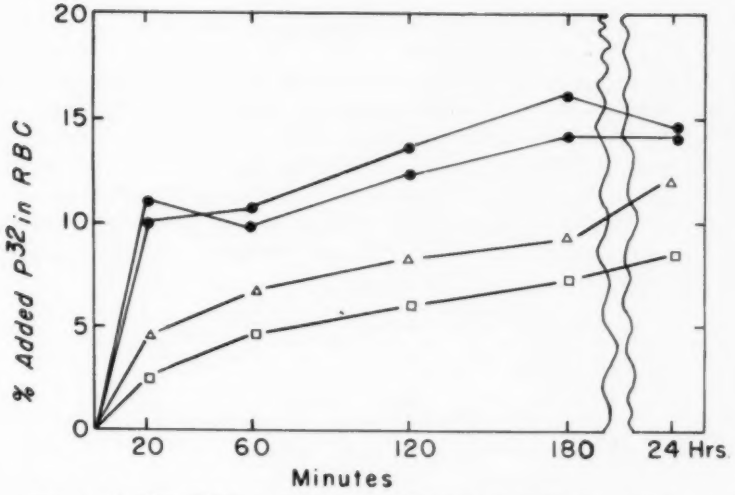

Fig. 4.—In vitro uptake of DFP^{32} by erythrocytes at room temperature. ● = DFP^{32} in propylene glycol (2 μg/ml. A.C.D. blood); △ = DFP^{32} in peanut oil (3 μg/ml. A.C.D. blood); □ = DFP^{32} in peanut oil (9 μg/ml. A.C.D. blood).

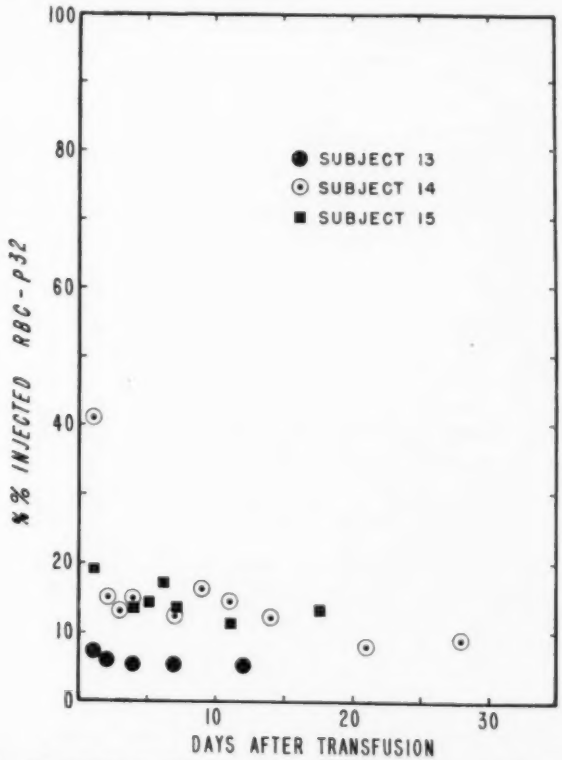

Fig. 5.—P^{32} activity in thrice washed erythrocytes following the transfusion of blood labeled in vitro with DFP^{32}. The P^{32} activity is plotted as a percentage of the activity in the erythrocytes of the 10-minute sample.

Red Cell Survival In Vivo After Tagging In Vitro With DFP^{32}.—In one subject (13), 10 minutes after a transfusion of whole blood tagged in vitro with DFP^{32}, 102 per cent of the injected red cell radioactivity was present in the recipient's circulation. This was determined by dividing the total injected c.p.m. contained in thrice washed erythrocytes from the transfused blood by the estimated total body hemoglobin of the recipient. The figure thus obtained was compared to the observed counts per minute per gram of circulating hemoglobin in the recipient 10 minutes postinjection.

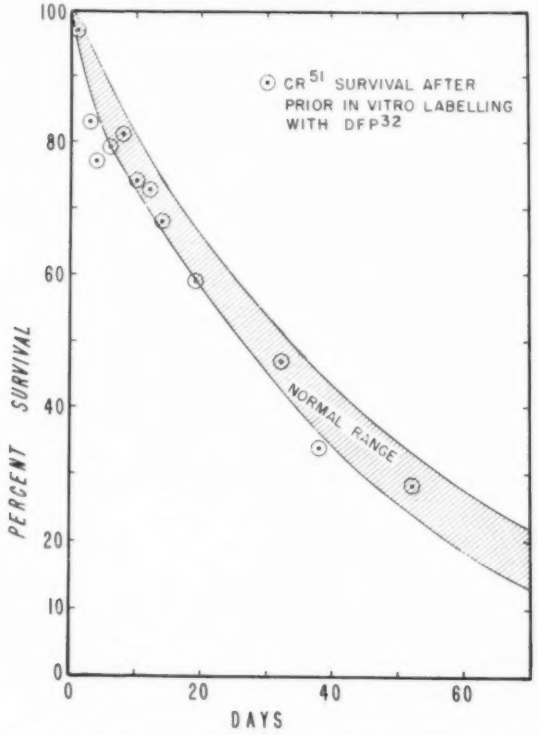

Fig. 6.—Cr^{51} survival of erythrocytes after prior 24-hour exposure to 2 μg. of DFP^{32} per milliliter of blood.

In Subjects 13 and 14 the erythrocytes were tagged for 4 hours at room temperature and in Subject 15 for 6 hours at room temperature and 18 hours at 5° C. In 3 subjects, during the first 24 hours after injection of in vitro tagged blood, there was a rapid decline of P^{32} in erythrocytes to values of from 10 to 20 per cent of the injected amount (Fig. 5). Forty-eight hours after injection the rate of decline of P^{32} activity in erythrocytes was about 2.1 per cent per day for Subject 13, 1.5 per cent per day for Subject 14, and 2.4 per cent per day for Subject 15. The rapid initial loss of radioactivity can be

explained either by rapid removal of tagged cells after transfusion or be "elution" of DFP^{32} from the circulating erythrocytes. In Subject 16, whole A.C.D. blood was labeled in vitro for 24 hours with 2.0 μg DFP^{32} per milliliter of A.C.D. blood and then tagged with sodium chromate^{51}. The chromium^{51} survival of these cells (Fig. 6) was normal, indicating that DFP^{32} does not alter the Cr^{51} survival of erythrocytes and that the initial rapid decline in P^{32} activity of these erythrocytes is most likely due to "elution" of P^{32} (from the circulating R.B.C.).

Excretion of Radioactivity From the G.I. Tract After the Ingestion of DFP^{32}-Labeled Blood.—Five normal volunteer medical students ingested 80 ml. of unwashed blood which had been tagged in vitro with 2.0 μg of DFP^{32} per milliliter of blood. Fourteen per cent of the added DFP^{32} was bound to the thrice washed erythrocytes. Stools collected for 6 days were analyzed for P^{32} content and 97.2 ± 9.7 per cent of the ingested radioactivity was recovered (Table I).

TABLE I. PER CENT RECOVERY IN 6-DAY STOOL COLLECTIONS OF NORMAL HUMAN BEINGS AFTER ORALLY ADMINISTERED UNWASHED DFP^{32}-TAGGED WHOLE A.C.D. BLOOD

Subject	16	17	18	19	20	Mean
Per cent recovery	106	107.6	88.1	84.6	99.6	97.2

DISCUSSION

Estimation of In Vivo Erythrocyte Survival by Intramuscular Injection of DFP^{32}.—During the first 5 or 10 days after intramuscular injection of DFP^{32}, the P^{32} content of circulating erythrocytes decreased at a rate faster than could be expected from red cell destruction alone. Thereafter the decrease occurred at a linear rate, 0.80 per cent per day. This strongly suggests that DFP^{32}, once affixed to erythrocytes in this manner, stays firmly bound after the first 5 to 10 days and that the P^{32} activity measured represents the survival of the erythrocytes. Our data and the previous work of others[1, 2, 3, 10] are in agreement with this interpretation. The reason for the early rapid decline of red cell DFP^{32} was not determined in these studies. DFP^{32} does label platelets[11, 12] and leukocytes.[12, 13] Since nearly all the platelets were removed in the preparation of the red cells for counting they are probably not responsible for the early rapid fall. We did not remove the leukocytes and cannot exclude the possibility that there was sufficient tagging of them to give what appeared to be an increased rate of red blood cell P^{32} decline in the first 5 to 10 days. The possibility that a small amount of P^{32} "elutes" from erythrocytes during this early period cannot be excluded either. Because of this early rapid decline in radioactivity of red cell P^{32}, it does not appear feasible to get an accurate estimate of red cell survival in vivo during the first 10 days after injection of DFP^{32}.

Binding of DFP^{32} to Red Cells In Vitro and the Survival of These Erythrocytes In Vivo.—Erythrocytes from A.C.D. blood are capable of binding approximately 0.8 μg of added DFP^{32} per milliliter of packed erythrocytes at 25° C. Most of this binding occurs within the first 4 hours, and no significant

additional labeling occurs during the next 20 hours. Erythrocytes exposed to DFP^{32} for 4 hours or 24 hours prior to transfusion show essentially the same rapid fall in red cell P^{32} activity after transfusion and the normal Cr^{51} survival of such erythrocytes strongly suggests that this initial fall is due to "elution" of DFP and not to erythrocyte damage.

Rate of Plasma Cholinesterase Turnover as Measured by DFP^{32}.—The initial rapid fall of radioactivity in plasma after intramuscular injection of DFP^{32} is quite similar to the rapid fall found after the injection of S^{35}- or I^{131}-labeled albumin.[9] It is probably due to mixing of the tagged proteins with the extravascular protein pool.[9] The second slower component appears to be exponential and is probably due both to the replacement of tagged proteins and to the elution of radioactivity from this protein since a similar half-life has been found for I^{131}-labeled albumin.[9] Others have shown that the half-life of biologically labeled S^{35} albumin[9] and gamma globulin[14] is 30 days.

Absorption of Radioactivity From the Gastrointestinal Tract After Feeding DFP^{32}-Labeled Blood.—In this study it was found that little, if any, P^{32} from orally administered DFP^{32}-whole blood mixture was absorbed from the gastrointestinal tract. Cohen and Warringa[3] have shown that only 5 per cent of the P^{32} from an intramuscularly injected dose of DFP^{32} can be recovered from the stools in 20 days. These two results suggest that the amount of blood lost in gastrointestinal hemorrhage may be quantitated with DFP^{32} by simultaneously measuring the P^{32} content of the patient's stools and blood.

Comparison of DFP^{32} With Sodium Chromate^{51} for the Determination of In Vivo Erythrocyte Survival Time.—DFP^{32} offers the following advantages over sodium chromate^{51} for the measurement of erythrocyte survival time. (1) After the first 10 days the rate of fall of P^{32} in erythrocytes is the same as that of transfused erythrocytes whereas Cr^{51} "elutes" from the circulating red cells at a rate of about 1 per cent a day.[6] (2) More efficient in vivo labeling takes place after the intramuscular injection of DFP^{32} than occurs after the intravenous injection of sodium chromate^{51}.[6]

Among the disadvantages of the DFP^{32} method are the following. (1) Under the conditions employed by us, DFP^{32} is far less effective for in vitro labeling than is sodium chromate^{51}. (2) The beta emission of P^{32} is more difficult to count than is the gamma emission of Cr^{51}. (3) The half-life of P^{32} (14 days) is shorter than the half-life of Cr^{51} (28 days). (4) The red cells must be washed with saline before radioactive assay to remove plasma radioactivity.

SUMMARY

1. After the intramuscular injection of 0.5 to 0.7 mg. of DFP^{32} into normal subjects, 21 to 41 per cent of the injected dose appeared in the circulating erythrocytes and 6 to 12 per cent in the plasma.

2. The rate of fall of radioactivity in erythrocytes was 0.80 per cent per day when measured over a 90-day period. A more rapid decrease of P^{32} in erythrocytes occurred during the first 10 days after intramuscular injection of DFP^{32} (1.9 per cent per day).

3. After intramuscular injection of DFP^{32}, a half-time of 16.6 days was found for the plasma P^{32} disappearance.

4. Erythrocytes exposed to DFP^{32} in vitro from 6 to 24 hours at 25° C. bound approximately 0.8 μg of DFP^{32} per milliliter of packed erythrocytes washed 3 times. Twenty-four hours after transfusion of these in vitro labeled erythrocytes, 90 per cent of the original content "eluted" from the circulating red blood cells.

5. Ninety-seven per cent of the radioactivity from orally ingested DFP^{32}-tagged blood was excreted in the feces.

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WHOLE BLOOD TRANSAMINASE LEVELS IN ANEMIA

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GLUTAMIC oxalacetic transaminase (GOT) activity has been reported to occur in both serum and whole blood of human beings.[1] No significant variations of whole blood activity, which is approximately 10 times greater than that of serum, could be found in a large number of pathological conditions.[1] To our knowledge, further study of this enzyme system in the blood of human beings has been limited to investigations on the effect of pyridoxine supplementation and deficiency on whole blood levels of this pyridoxine-linked enzyme.[2, 3]

During a recent investigation on the effect of isonicotinic acid hydrazide on whole blood transaminase activity,[4] marked variations of GOT activity were noted in patients with certain anemias.[20] This report is concerned with the presentation of data on these variations during periods of increased marrow activity associated with red cell proliferation. The results suggest that alterations in the average age of the circulating erythrocytes are accompanied by changes in the transaminase activity of the whole blood.

EXPERIMENTAL

Subjects.—The patients selected for this study were separated into 3 categories. The first, Group A, served as a control group and consisted of a series of patients with a variety of medical and surgical complaints. No hematologic abnormalities were demonstrated in this group. The second, Group B, served as a control on the effect of anemia per se on GOT activity. This group was composed of patients with chronic anemia unassociated with hemorrhage or hemolytic episodes. Included in this category were several cases of megaloblastic anemia as well as two cases whose only demonstrable abnormality was sickle-cell trait. The third, Group C, was composed of a series of patients with hemolytic anemia including sickle-cell anemia, thalassemia, congenital spherocytosis, plumbism, and acquired hemolytic anemia secondary to a variety of disorders.

Laboratory Methods.—Blood was collected into dry balanced oxalate[21] and a 1:20 dilution was prepared in distilled water. The diluted whole blood was centrifuged at 2,500 r.p.m. for 15 minutes to remove particulate matter. The clear supernatant was analyzed for transaminase activity routinely on the same day. In several cases, where necessary, this hemolyzate was stored at –20° C. for several days prior to analysis. Its hemoglobin content was also determined and all GOT values were calculated on the basis of an arbitrary 100 mg. hemoglobin to provide a rough comparative measure.

A modification[5] of the spectrophotometric procedure developed by Karmen[6] was used for the estimation of the glutamic oxalacetic transaminase activity. For this assay, a 0.2 to 0.3 ml. aliquot of the 1:20 dilution of whole blood was analyzed. Readings were

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taken in a Beckman D.U. spectrophotometer, one unit representing an optical density change of 0.001 O.D. unit per minute, in a total volume of 3.0 ml. under the conditions specified. All operations were carried out at room temperature. The temperature coefficients developed by Steinberg and associates[7] were found to be satisfactory in our hands and were used to correct the values to a standard temperature of 25° C. Plasma GOT levels were carried out in the same manner using 0.2 to 0.5 ml. aliquots of undiluted plasma obtained from the same oxalated specimen of blood.

Hemoglobin was determined by estimating oxyhemoglobin content at 540 mμ in the Beckman spectrophotometer. This instrument was standardized for hemoglobin by the manometric procedure of Van Slyke and Neill.[8]

RESULTS

Although the term "whole blood transaminase" has been used throughout this study there is evidence to indicate that the major portion of this GOT activity resides in the red cells. Table I compares the GOT activity of whole blood with that of washed erythrocytes. It seems clear that, in normal blood, the enzyme activity contributed by both the plasma and the components of the "buffy" coat is insignificant. The serum GOT values of the patients studied in this investigation were within the normal range of 9 to 40 units per milliliter. Although subsequent studies were done on dilutions of whole blood for convenience, the values reported reflect the GOT activity of the red cell contents.

TABLE I. GOT ACTIVITY OF WASHED RED CELLS VERSUS WHOLE BLOOD

SAMPLE	GOT UNITS/100 MG. HEMOGLOBIN	
	WASHED CELLS	WHOLE BLOOD
1	390	440
2*	1,770	1,700
3	480	470
Mean	880	870

*Sample from a patient with acquired hemolytic anemia.

The GOT activity in the blood of a group of 22 patients (Group B) with mild to severe chronic anemia was studied to ascertain whether or not the range of blood transaminase values was consistent with that obtained in a series of hematologically normal patients (Group A). An additional group of 19 patients (Group C) with a variety of hemolytic anemias was also studied. A comparison of the mean GOT activities of Group C with those of both normal patients (Group A) and patients with simple anemias (Group B) is presented in Table II.

The results indicate that the mean transaminase activity for the group of patients with hemolytic anemias (Group C) is approximately 2 to 2½ times greater than the mean of either of the two control groups. Statistical evaluation indicates that there is a strong probability that the observed differences are not due to chance. Perhaps even more interesting is the fact that there is no significant overlapping of the range of GOT activity of the experimental group (Group C) with that of either of the control groups. In only one case

was a value obtained that did not fit within the range of its group. This result occurred in a patient who was at first presumed to have an acquired hemolytic anemia secondary to carcinoma (See footnote [†], Table II). Bone marrow aspiration indicated the presence of malignant cells with no evidence of erythroid hyperplasia. Reticulocytosis was absent.

TABLE II. MEAN BLOOD GOT LEVELS IN PATIENTS WITH AND WITHOUT HEMATOLOGIC DYSCRASIAS (± S.E.)

GROUP	NO.	MEAN HEMOGLOBIN (GM. PER CENT)	MEAN GOT (UNITS/100 MG. HB.)	"P"
A (Normal)	23	13.2	526 ± 20 (320 – 780)*	0.33
B (Chronic anemia)	19	9.0	507 ± 19.6 (320 – 720)	> 0.01
C (Hemolytic anemia)	18	8.9	1,300 ± 25.6 (860 – 2,500)†	

*Range of values indicated by parentheses.
†One value of 400 units per 100 mg. hemoglobin omitted from this range but not from the mean.

As expected, reticulocyte counts in all of the patients in the group with hemolytic anemia (Group C) were elevated. Although an attempt to correlate the reticulocyte count with the level of transaminase activity in the blood was unsuccessful, further studies were carried out to determine the role of reticulocytes on the increase in blood transaminase activity. Oxalated blood containing an elevated number of reticulocytes was centrifuged in a capillary tube. The plasma and buffy coat were removed and the red cell layer was divided into 3 equal parts: top, middle, and bottom. Each section was reconstituted with an equal portion of its own plasma, and reticulocyte counts and GOT assays were done on each fraction. The results (Table III) indicate a rough correlation between the degree of reticulocytosis and the level of transaminase activity.

TABLE III. TRANSAMINASE CONTENT OF VARIOUS FRACTIONS OF CENTRIFUGED ERYTHROCYTES

EXPERIMENT NO.	FRACTION	GOT UNITS/100 MG. HB.	PER CENT RETICULOCYTES
1	Top	1,800	30.0
	Middle	1,080	9.8
	Bottom	720	5.1
2	Top	1,560	28.8
	Middle	1,130	14.1
	Bottom	790	5.0

In an attempt to ascertain the temporal relationship between the appearance of elevated blood GOT values and the appearance and degree of reticulocytosis, hemolytic anemia was produced in two rabbits by the intraperitoneal injection of 125 mg. of phenylhydrazine. Repeated samplings of blood, obtained by cardiac puncture, were analyzed for both GOT activity and hemoglobin. Reticulocyte counts were also done on the same blood samples. The

data, presented in Fig. 1, demonstrate that an increase in transaminase activity does occur shortly after the peak in the reticulocyte response. Both animals, however, showed no more than a slight increase in GOT activity at the time when reticulocytosis was maximal. Furthermore, in Rabbit 16 (Fig. 1), the transaminase level did not rise until after the reticulocyte count had dropped sharply. These relationships suggest that reticulocytes themselves may not be entirely responsible for the marked increase in enzyme activity noted in these animals.

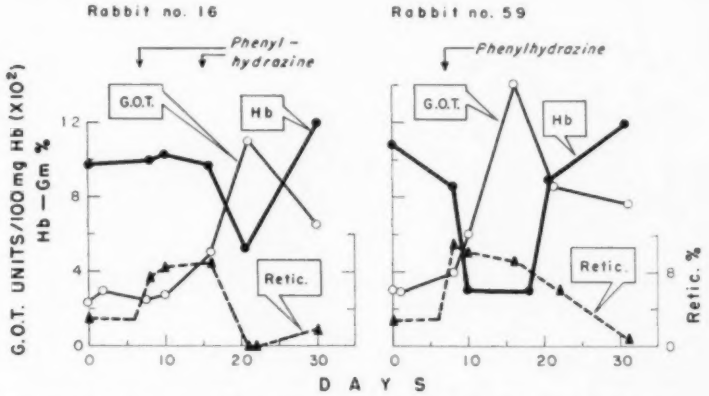

Fig. 1.—Serial GOT levels in rabbits after phenylhydrazine induced hemolytic anemia.

Preliminary data are available on serial studies in several patients. One with sickle-cell anemia, 2 with acquired hemolytic anemia, and 2 with megaloblastic anemia were studied prior to and during a course of therapy. In both cases of hemolytic anemia the elevated transaminase activities, obtained shortly after steroid therapy had been instituted, remained high despite the drop in reticulocyte count and the return of the hemoglobin to normal (Fig. 2). Although no intermediate values were obtained, the elevated GOT values in these patients had dropped to normal some 100 to 130 days after the start of therapy. Subsequent rises seem to be coincident with the tapering off of the administration of steroids.

In the two patients with megaloblastic anemia, normal transaminase values were obtained prior to therapy (Fig. 3). Shortly after B_{12} therapy was instituted a peak reticulocytosis occurred together with a sharp elevation in GOT activity. Subsequent transaminase values remained elevated despite the drop in reticulocyte count and the return of hemoglobin to nearly normal levels. The return of the elevated GOT activities to normal was slow in both patients. In one, J. K., a significant supplementary rise in enzyme activity occurred some 10 days after the patient was started on oral iron therapy. No reticulocytosis could be demonstrated at this time.

In the patient with sickle-cell anemia (Fig. 4) transaminase levels were variably elevated, although minimally. There was no consistent trend in

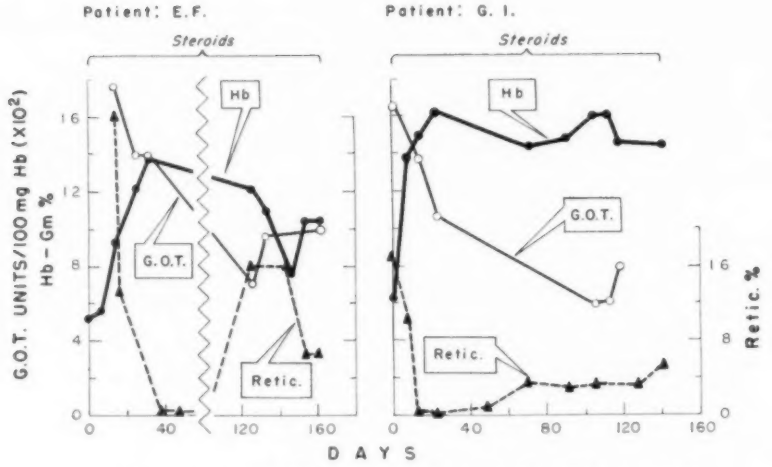

Fig. 2.—Serial GOT levels in two cases of acquired hemolytic anemia.

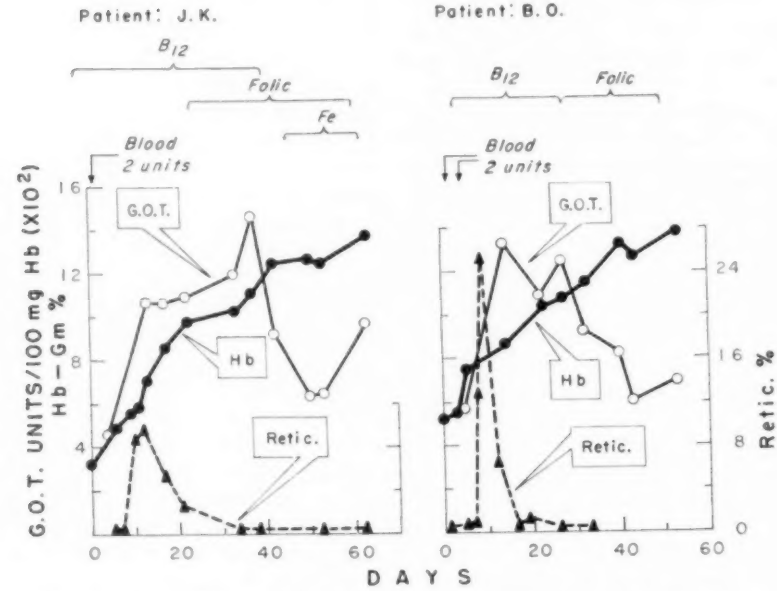

Fig. 3.—Serial GOT levels in two cases of megaloblastic anemia during therapy.

either direction during the period studied. Similar variations in GOT activity were noted in one other case of sickle-cell anemia (data not presented).

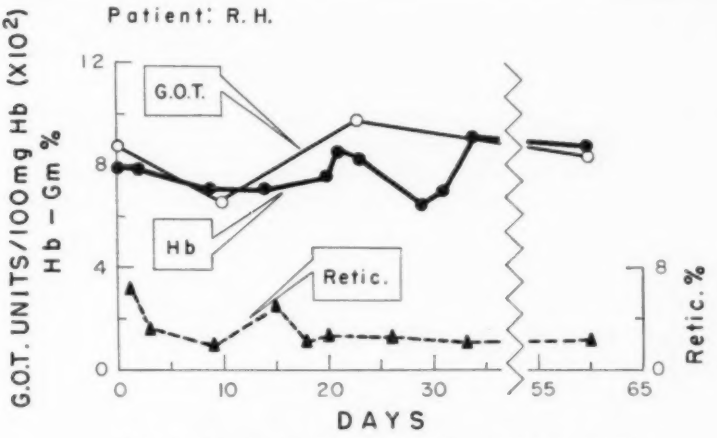

Fig. 4.—Serial GOT levels in a case of sickle-cell anemia.

The increased blood GOT activity observed in patients with anemia during periods of bone marrow hyperactivity and increased red cell proliferation suggested that similar findings could be expected in patients with anemia secondary to chronic hemorrhage. The data in Table IV indicate that in the greater majority of such patients blood transaminase values are elevated and within the range of patients with hemolytic anemia (Group C). Further study is necessary to relate this increased GOT activity to onset, duration, and severity of the blood loss.

TABLE IV. BLOOD GOT ACTIVITY IN PATIENTS WITH ANEMIA SECONDARY TO CHRONIC HEMORRHAGE

SUBJECT	HEMOGLOBIN (GM. PER CENT)	GOT (UNITS/100 MG. HB.)
1*	6.3	2,300
2	4.1	1,980
3	5.3	1,820
4	9.6	1,800
5	6.9	1,780
6	6.7	1,440
7	6.0	1,370
8*	7.3	1,280
9	8.2	1,100
10	8.2	1,050
11	7.1	900
12	7.0	720
13	8.6	660

*Severe iron deficiency.

DISCUSSION

The markedly increased mean whole blood transaminase activity (Table II) observed in the group of patients with hemolytic anemia (Group C), both

congenital and acquired, cannot be correlated with the degree of anemia. The highest value obtained, in a case of plumbism in a child, was associated with a minimal anemia. Many of the lower GOT values in both groups with anemia (Groups B and C) were associated with moderate to severe anemia. Furthermore, the mean hemoglobin values for both of these groups are almost identical despite the marked difference in mean GOT activity.

Sufficient data are not available to establish the reason for the increased level of GOT activity in the blood of patients with hemolytic anemia. It is possible that immature and young cells contain larger amounts of transaminase enzyme and that the observed increased GOT activity is a reflection of a younger erythrocyte population. The finding that the transaminase activity in the blood of the two patients with hemolytic anemia declined slowly toward normal as the hemolytic process subsided is consistent with the above interpretation. No such correlation could be demonstrated in sickle-cell anemia (Fig. 4).

The observations on the two patients with megaloblastic anemia before and during therapy provide corroborative evidence that marked erythroid response of the bone marrow and the consequent change in the average age of the circulating red cells influence the over-all transaminase activity of the blood. It is clear from these studies that reticulocytes are not wholly responsible for the changes observed. Blood GOT levels in these patients (Fig. 3) remained elevated despite the return of the reticulocyte count and the hemoglobin to normal or nearly normal.

It would appear that blood transaminase activity offers promise as an indicator of the influx of red cells into the peripheral circulation. A number of attempts have been made in the past to measure red cell proliferation in some terms other than reticulocyte response. Glycolytic activity of red cells, found to be increased in reticulocytes, has been suggested as a rough measure of marrow response.[15] Blood cholinesterase activity, similarly increased in reticulocytes,[16] has been found to be increased in megaloblastic anemia after therapy[17-19] as well as in anemia due to blood loss.[16, 18] There have been indications, however, that this increase is frequently not seen in cases of hemolytic anemia or massive hemorrhage despite other indications of bone marrow hyperactivity.[17] Blood transaminase activity, on the other hand, is more consistently elevated in hemolytic anemia (Table II) as well as after severe hemorrhage (Table IV).

The physiologic significance of the presence of an elevated level of transaminase activity in reticulocytes and, presumably, in young red cells needs to be clarified. These findings may indicate that transaminase enzymes play an important role in normal erythropoiesis. On the other hand, the apparent decrease in the GOT activity of red cells as they mature may simply reflect the slow destruction of many enzyme systems as a natural consequence of aging.[9] Sufficient experimental evidence is available, however, to indicate that a defect in hemoglobin synthesis occurs in animals[10-12] and in man[13, 14] in association

with a deficiency of pyridoxine, the coenzyme of transaminase. This interference with hemoglobin synthesis could be mediated through the effect of pyridoxine deficiency on the tissue level of one or more of the pyridoxine-linked transaminases. Further study is being carried out to clarify these relationships.

SUMMARY

1. Whole blood GOT levels of patients with hemolytic anemias and anemia secondary to chronic hemorrhage have been shown to be approximately twice as high as that of normal controls.

2. Good correlation between reticulocyte counts and GOT levels has been demonstrated in various layers of centrifuged blood.

3. In two cases of acquired hemolytic anemia, GOT levels have been shown to be markedly elevated, slowly returning toward normal on steroid therapy. No such trend was noted in sickle-cell anemia.

4. Normal GOT levels, obtained in two cases of melagoblastic anemia increased markedly after B_{12} therapy. They were maintained at this level despite the return of the reticulocyte count and hemoglobin to normal. Subsequent determinations indicated a slow decline toward normal values over a period of 30 days.

5. It has been suggested that measurements of whole blood GOT activity may be useful as an indicator of bone marrow activity.

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SERIAL SERUM GLYCOPROTEIN AND PROTEIN STUDIES IN PULMONARY TUBERCULOSIS*

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CHANGES in serum proteins and glycoproteins have been studied in a number of physiologic and pathologic states in both human beings and animals.[1, 2] Elevation of glycoproteins in infections and especially tuberculosis has been well documented.[3-8] In a recent publication[7] we reported glycoprotein and protein studies on 56 tuberculous patients shortly after their admission to the hospital. Plasma samples were subjected to the Lever modification of Cohn's fractionation technique and a determination was made of the glycoprotein and protein contents of each fraction and of the seromucoid fraction. These results were correlated with the x-ray and clinical data. It was found that there was an increase in glycoprotein concentration in all fractions in far-advanced disease, and that this increase was roughly proportional to the degree of *pneumonic exudation* and the *extent* of the disease. This was most noticeable in the seromucoid fraction. Fresh, exudative disease showed higher levels than the more fibrotic variety, and far-advanced cases showed higher levels than minimal or moderate cases.

In order to further investigate the glycoprotein changes in tuberculosis, serial analyses were undertaken during the course and treatment of the disease. A similar serial analysis of the protein changes by paper electrophoresis was recently reported by Leggat.[9] For the present study, a serum specimen from newly hospitalized patients with extensive exudative tuberculosis was analyzed for total proteins, total glycoprotein, seromucoid, gamma globulin protein, and gamma globulin polysaccharide. The same analyses were repeated at intervals of 3 to 6 months during the hospitalization. This study includes also the protein and glycoprotein levels in 85 normal, healthy medical students and laboratory technicians who were used as controls and establishes a set of normal values by this technique. Representative case reports are also added.

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*Due to the lack of a generally accepted nomenclature for the serum glycoproteins, it is advisable to define the terms employed: *Total serum glycoprotein:* The protein-bound hexoses of the serum proteins precipitated by an ethanol concentration of 90 per cent by volume at 25° C. (expressed as mg. per 100 ml. serum). *Seromucoid:* The hexose moiety of the perchloric acid-soluble, phosphotungstic acid-insoluble proteins occurring in serum (expressed as mg. per 100 ml. serum). *Gamma globulin polysaccharide:* The hexose components of the protein-bound carbohydrates of the gamma globulin fraction (expressed as mg. per 100 ml. serum). It should be noted that an attempt has been made recently to establish uniformity of terminology.[2]

MATERIALS AND METHODS

Twenty milliliters of blood was obtained by venipuncture, allowed to clot, and the serum separated by centrifugation. The serum was then quickly frozen and stored at −20° C. until the chemical determinations were performed. Total serum protein was determined by the biuret method of Weichselbaum.[10] Total serum glycoprotein and seromucoid were determined by the procedures of Weimer and Moshin.[6] The gamma globulin fraction was isolated by a modification[11] of the method of Nichol and Deutsch. The protein and polysaccharide components of the fraction were estimated by biuret and orcinol reactions, respectively.[6, 10, 11]

For the purpose of this study 35 patients were selected. One group was comprised of 19 patients who showed a favorable response to medical therapy. A second group contained 10 patients who showed little change during their hospitalization; their disease was either chronic though active on admission or subacute because of 2 to 3 months' therapy prior to admission to this hospital. A third group consisted of 6 patients with progressive disease.

RESULTS

Normal values are included in Table I. Although based on a much larger group, total serum glycoprotein, seromucoid, and total serum protein concentrations are in good agreement with those previously reported.[7] Gamma globulin polysaccharide and protein values are appreciably higher. The divergence is due presumably to the presence of beta globulin in the precipitate obtained by an adaptation[11] of the procedure of Nichol and Deutsch.

Patients Responding Satisfactorily to Original Treatment (Table I).—These patients were selected from our routine admissions because of their extensive, acute, exudative disease and were treated with a bed rest regimen and specific chemotherapy (streptomycin, isoniazid, para-aminosalicylic acid). All showed improvement clinically, bacteriologically, and radiologically.

The values of the serum fractions, with the exception of the total protein, were uniformly high at the beginning of treatment. The total serum glycoprotein was 157 mg. per cent (44 per cent elevated), gamma globulin protein 2.5 Gm. per cent (31 per cent elevated), gamma globulin polysaccharide 49 mg. per cent (32 per cent elevation), and seromucoid 27 mg. per cent (125 per cent elevation). The seromucoid fraction was apparently the most affected by acute disease.

With treatment of the tuberculosis the values of all the protein fractions tended to return to normal. The degree of improvement in the various fractions was roughly parallel; it was most marked during the first 3 months, and the values approached normal at 6 months.

It was observed also that the cultures for *Mycobacterium tuberculosis* tended to become negative before the end of the 6 months, while the x-ray improvement continued long after this time.

Chronic Cases (Table I).—These patients either had a term of treatment at another hospital prior to their transfer here and hence were subacute on admission, or else they were patients who had chronic disease for some time. The course of their disease during hospitalization was such that inactivity of the disease was not achieved but smoldered along with the sputum usually

positive for acid-fast bacilli and serial studies showing evidence of activity. The symptoms varied from mild to moderately severe and consisted mainly of cough, expectoration, and malaise.

TABLE I. GLYCOPROTEIN AND PROTEIN VALUES INCLUDING THE STANDARD DEVIATION OF THE MEAN IN NORMALS, PATIENTS SHOWING SATISFACTORY IMPROVEMENT, AND CHRONIC PATIENTS

CLASSIFICATION	TIME (MONTHS)*	TOTAL SERUM GLYCOPROTEIN (MG. %)	SEROMUCOID FRACTION (MG. %)	GAMMA GLOBULIN POLYSACCHARIDE (MG. %)	TOTAL SERUM PROTEIN (GM. %)	GAMMA GLOBULIN PROTEIN (GM. %)
Normal	(85)	109 ± 1.0	12 ± 0.3	37 ± 0.6†	7.3 ± 0.06	1.9 ± 0.03†
Satisfactory improvement	0 (19)	157 ± 5.1	27 ± 2.1	49 ± 2.4	7.0 ± 0.13	2.5 ± 0.13
	3 (8)	133 ± 6.7	17 ± 1.7	43 ± 2.6	7.1 ± 0.20	2.3 ± 0.16
	6 (19)	116 ± 3.5	13 ± 1.1	40 ± 1.6	7.0 ± 0.12	2.1 ± 0.09
	12 (6)	110 ± 2.1	12 ± 1.7	39 ± 2.0	6.9 ± 0.20	2.0 ± 0.16
Chronic with previous treatment	0 (10)	111 ± 4.0	14 ± 1.4	37 ± 1.5	7.3 ± 0.14	2.0 ± 0.10
	6 (9)	110 ± 5.7	11 ± 0.8	38 ± 2.6	7.3 ± 0.17	2.0 ± 0.10

*Numerals in parentheses refer to the number of cases.
†80 individuals.

All values in these cases were either in the normal range or close to it. After 6 months of treatment with a modified bed rest regimen and antibiotic therapy, there was no significant change in their protein levels.

Progressive Cases (Table II).—Six patients with progressive disease were chosen: of these, one died at this hospital (Case 2), 3 were transferred to other hospitals or discharged in a terminal condition, and 2 have had a generally progressive course with occasional remissions in their disease and, at this time, are still alive.

TABLE II. GLYCOPROTEIN AND PROTEIN VALUES IN PATIENTS WHOSE DISEASE PROGRESSED (ON ADMISSION AND AFTER 6 MONTHS' THERAPY)

	TOTAL SERUM GLYCOPROTEIN (MG. %)		SEROMUCOID FRACTION (MG. %)		GAMMA GLOBULIN POLYSACCHARIDE (MG. %)		TOTAL SERUM PROTEIN (GM. %)		GAMMA GLOBULIN PROTEIN (GM. %)	
PATIENT	(1)*	(2)†	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
T. W.‡	195	197	57	59	52	51	6.4	6.4	2.9	2.6
M. M.	114	142	11	28	40	44	7.7	5.9	2.5	2.0
J. B.	140	167	21	24	39	43	7.0	7.5	2.1	2.2
D. C.	144	134	17	15	48	39	7.8	6.9	2.5	2.1
J. J.	90	92	10	8	25	28	7.0	6.9	1.6	1.7
J. M.	100	108	10	8	39	39	7.5	8.0	2.3	2.2

*On admission (1).
†After therapy (2).
‡Reported in detail in text.

The chemical values showed interesting results. In none of the cases was there any marked abnormality in the total protein levels. The fractions showed two types of values. Four of the patients had markedly elevated values

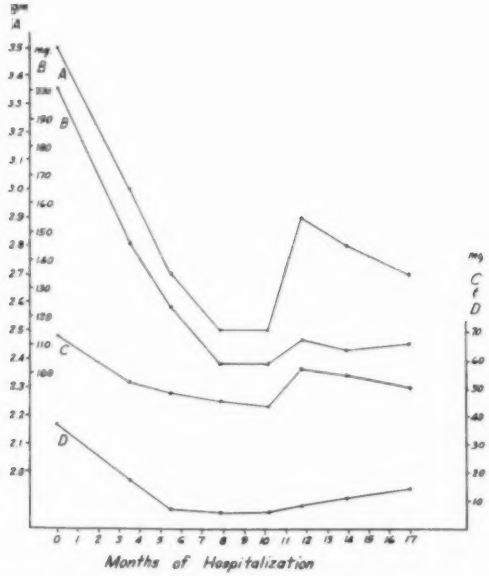

Fig. 1.—Various glycoprotein and protein levels in a patient responding to treatment for pulmonary tuberculosis (Case 1). *A* = gamma globulin protein. *B* = total glycoprotein. *C* = gamma globulin polysaccharide. *D* = seromucoid.

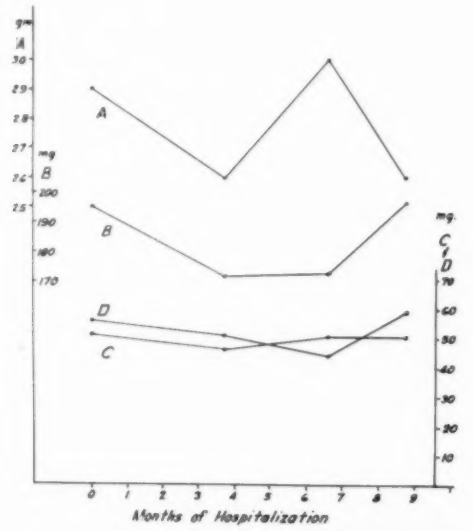

Fig. 2.—Levels in a patient with progressive tuberculosis who succumbed to the disease after 9 months' hospitalization. *A* = gamma globulin protein. *B* = total glycoprotein. *C* = gamma globulin polysaccharide. *D* = seromucoid.

on admission, and they remained elevated as the disease progressed. The other two patients, however, entered the hospital with normal levels of total glycoprotein, seromucoid, and gamma globulin polysaccharide and, despite an increase in their disease, these values remained at normal levels. In general, again, the seromucoid paralleled the total glycoprotein levels, as well as the gamma globulin polysaccharide.

CASE REPORTS

CASE 1.—(Clinical improvement, Fig. 1.) A 29-year-old white man (E. C.) had pulmonary tuberculosis diagnosed in August, 1954. The chest x-ray showed minimal infiltration in the left mid-lung field and far-advanced exudative disease on the right with atelectasis and cavitation; the sputum was positive for *Myco. tuberculosis*. The patient was placed on bed rest regimen and treated with streptomycin, para-aminosalicylic acid, and isoniazid. The last positive gastric culture was obtained 5 months after beginning treatment and it remained negative after that except for one positive culture 15 months after the start of treatment. The gamma globulin protein showed a level of 3.5 Gm. per cent on admission, dropped to 2.5 Gm. per cent after 8 months, then varied between this and 2.9 Gm. per cent for the next 7 months; thus, after 17 months it still showed an abnormal elevation. The initial value for total glycoprotein was 201 mg. per cent and dropped to normal levels at 8 months. The gamma globulin polysaccharide was originally 68 mg. per cent and at the end of 17 months remained at the high figure of between 50 mg. and 60 mg. per cent. The seromucoid started at 37 mg. per cent and dropped to normal levels at 6 months. Thus the gamma globulin and its polysaccharide component remained elevated and perhaps could be considered in this case a sensitive index of activity paralleling the x-ray picture. The roentgenogram showed rapid favorable change for the first 8 months and then continued to show slower favorable change for the next 16 months.

CASE 2.—(Progressive disease, Fig. 2.) A 61-year-old white man (T. W.) had tuberculosis for about 6 months prior to admission. For the preceding 12 years he had also had diabetes mellitus and took insulin regularly. The sputum contained tubercle bacilli. X-rays showed considerable bilateral exudative disease, predominantly on the right with extensive cavitation. The patient was placed on bed rest, streptomycin, para-aminosalicylic acid, and isoniazid. His tuberculosis did not respond and the disease progressed. Diabetic gangrene developed in the left foot which also responded poorly to treatment. He died of a cerebral embolus 9 months after entry.

This patient's sputum remained positive for *Myco. tuberculosis* and his x-ray showed steady progression and extension of his tuberculosis. The serum protein values all remained highly elevated and reflected the clinical progress and systemic toxicity.

CASE 3.—(Chronic disease, Fig. 3.) This patient was a 42-year-old Chinese man (W. W.) who had a diagnosis made of tuberculosis of the lung and cervical lymph nodes 3 years prior to admission. X-ray at that time showed chronic productive disease of both pulmonary apices. The sputum was positive for acid-fast bacilli. He was placed on modified bed rest regimen and was treated with streptomycin, para-aminosalicylic acid, and isoniazid. Six months after admission to the hospital he had a sudden massive pulmonary hemorrhage and died.

His sputum was usually negative but occasionally positive for *Myco. tuberculosis*. X-rays showed little change during this time, but had previously shown improvement and had relapsed. The protein studies showed the gamma globulin protein to be somewhat high on admission but it dropped to within normal limits shortly before his death. The total glycoprotein was normal on admission but revealed a mild but definite elevation before the terminal episode. High normal levels were evident in the gamma globulin polysaccharide

and the seromucoid. In this case the total serum glycoprotein level provided the best chemical index of activity. Since the patient showed no systemic toxicity of any note it was not surprising to find chemical values normal or near normal.

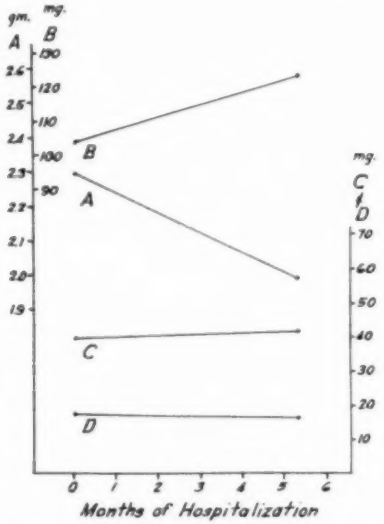

Fig. 3.—Various glycoprotein and protein levels in a patient with chronic disease (Case 3). *A* = gamma globulin protein. *B* = total glycoprotein. *C* = gamma globulin polysaccharide. *D* = seromucoid.

DISCUSSION

As previously reported,[7] the most consistent change in the protein and glycoprotein relationships in pulmonary tuberculosis is the elevation of the total serum glycoprotein and seromucoid levels in patients with extensive pneumonic tuberculosis. This report further substantiates the finding to show the importance of very early examination of the blood, as the chemical values drop rapidly during the first 3 months of treatment. If, as happened in many of our cases, the patient had had antituberculous therapy prior to admission, the values will have fallen to within relatively normal limits, even though the roentgenogram still shows evidence of an exudative lesion.

For the sake of brevity, detailed results of all the cases showing clinical improvement are omitted. With a few exceptions the total serum glycoprotein and seromucoid levels exhibited a consistent decline as healing occurred.

In the 6 cases with progressive disease, the first 4 had elevated total glycoprotein on admission. These remained elevated in spite of treatment to which the disease did not respond. Two cases had low levels on admission even though their tuberculosis was extensive and pneumonic. These are the only two cases with much exudation that we have seen with low levels. Perhaps in those rare cases in which this condition is found, the test can be utilized as a sign signifying a poor prognosis.

It is hoped that these findings may preface further investigations to determine the physiopathologic relationships of the protein-bound carbohydrates of serum in infections.

SUMMARY

The total serum protein, total serum glycoprotein, seromucoid, serum gamma globulin protein, and its polysaccharide component were determined in 85 normal controls and on 35 patients on admission to the hospital and at 3- to 6-month intervals following hospitalization. These were classified on the basis of their response to treatment as "satisfactory improvement," "chronic," and "progressive disease." The chemical values were correlated with their hospital course. Those patients with retrogressive disease showed an elevation on admission and a decline toward normal of the seromucoid, total glycoprotein, gamma globulin, and gamma globulin polysaccharide. The chronic patients exhibited little change during their hospitalization. Those patients whose disease progressed during treatment fell into two groups. Four had elevated levels on admission and they remained high throughout treatment; 2 had low levels which remained low. Speculation was made as to the prognostic significance of this finding.

The technical assistance of Jean Redlich-Moshin and Hisako Nishihara is gratefully acknowledged.

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STUDIES WITH INAGGLUTINABLE ERYTHROCYTE COUNTS

VII. Further Investigation of the Hemolytic Mechanism in Untreated Pernicious Anemia and the Demonstration of a Hemolytic Property in the Plasma

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IN RECENT years more refined techniques of differential agglutination and of radioisotopic labeling of erythrocytes have been used to investigate the role of hemolysis in Addisonian pernicious anemia. Loutit,[1] and Singer, King, and Robbin[2] showed that erythrocytes from persons with untreated pernicious anemia had short survival times when transfused to normal persons. London and associates[3] demonstrated a shortened lifespan of pernicious anemia erythrocytes by labeling the patient's cells with glycine containing radioactive nitrogen. Singer and co-workers[2] demonstrated that erythrocytes from a patient with treated pernicious anemia in remission survived normally in a normal person. In 1954, Hamilton and his associates[4] demonstrated that normal red cells in patients with severe pernicious anemia in relapse had a shortened survival time. Mathematical analysis of their data indicated that hemolysis contributed to the anemia and the rate of erythropoiesis failed to accelerate. The accelerated destruction of normal erythrocytes in patients with untreated pernicious anemia suggested the presence of a hemolytic factor in the disease so that the shortened survival of untreated pernicious anemia cells was not due entirely to a developmental abnormality of the erythrocytes.

The purpose of the current studies was to make further inquiry into the hemolytic mechanisms of pernicious anemia and to determine whether or not a hemolytic property could be found in the plasma of patients with untreated pernicious anemia.

METHODS

Transfusions.—The data for analysis were obtained by the Ashby technique of differential agglutination of erythrocytes as described by us.[5, 6] The donor's blood was collected from healthy young men or patients with severe untreated pernicious anemia. The donor's blood was of group O and was transfused to recipients of group A or B with compatible Rh subtypes. Anti-A or anti-B serum was used to agglutinate the recipient's cells,

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leaving the donor's cells free for counting. In experiments involving two or more recipients, one of whom also served as a donor, ON or AN cells were transfused to an AMN recipient and anti-M serum was used to agglutinate the recipient's cells. Total erythrocyte counts of the recipient's blood were made coincidentally with the inagglutinable cell counts.

Five hundred milliliters of donor blood was collected in 3.2 per cent solution of sodium citrate ($Na_3C_6H_5O_7 + 2\ H_2O$), and stored less than 18 hours at 4° C. The supernatant plasma was removed and the cells were washed once with cold 0.9 per cent NaCl solution. Erythrocyte counts of the donor's blood were made and a measured volume of blood was transfused into the recipient by gravity.

In the 24 hours following each transfusion, the serum samples from the recipients contained no abnormal amounts of hemochromogens or bilirubin. At the end of a survival study the indirect Coombs' test, using fresh donor's cells and recipient's serum, was negative (except as specified in Experiment 2).

Incubation Studies Before Transfusion.—In Experiments 4 and 5, the donor's blood was collected, immediately centrifuged, and the supernatant plasma removed. The cells were then transferred to freshly prepared plasma, from either a normal person or a patient with untreated pernicious anemia. The plasma-cells mixture was stored at 4° C. for 14 hours and then placed at room temperature for 4 hours. The suspension was centrifuged, the supernatant plasma removed, and the red cells washed with cold 0.9 per cent NaCl solution. The erythrocytes were immediately transfused into a normal recipient. When cells were stored in a foreign plasma, the erythrocytes were of group O and the plasma was from normal group O or A blood of the compatible Rh subtype. In the incubation studies the ratio of packed cells to plasma was approximately 1:2 and the volume of packed cells transfused ranged from 125 ml. to 250 ml.

TABLE I. CLINICAL AND LABORATORY DATA IN PATIENTS STUDIED

EXPERIMENT	CASE 1 EXPER. 2, FIG. 1 (LOWER CURVES)	CASE 2 EXPER. 4, FIG. 3	CASE 3 EXPER. 5, FIG. 4
Sex and age	F. 83	M. 80	M. 50
Duration of symptoms	1 yr.	5 mo.	1 yr.
Achlorhydria (with histamine stimulation)	Present	Present	Present
Initial erythrocyte count 10^6/mm.^{3}	0.99	1.43	0.98
Initial leukocyte count	6,400	6,400	1,550
Initial platelet count	80,000	67,000	36,000
Spinal cord disease	Moderate	Slight	None
Megaloblastic bone marrow	Yes	Yes	Yes
Maximum reticulocyte count in days after B_{12} therapy	17.6 per cent (eighth)	5.8* (fifth)	15 per cent (seventh)
Absence of intrinsic factor (Schilling test)	Not done	Not done	Yes
Evidence of renal disease or occult blood in stool	None	None	None
Maximum erythrocyte count after B_{12} therapy (10^6/mm.^{3})	4.2	3.69	4.4
X-ray study upper and lower G.I. tract normal	Yes	Yes	Yes

*Intranasal instillation of B_{12} was started several days before intramuscular B_{12} therapy. This resulted in the low reticulocyte count reported here.

The Patients.—The patients had the characteristic clinical and laboratory findings of Addisonian pernicious anemia. They were severely anemic and in relapse. The significant data for patients used in Experiments 2, 4, and 5 are included in Table I. The data for Experiments 1 and 3 were presented in a previous publication as cases 9 and 10.[4]

Method of Analysis.—In these studies the inagglutinable erythrocyte counts are direct counts of the biologically tagged red cells transfused to a recipient, Table II. The observed counts were adjusted[7] by adding the number of cells lost each day by normal aging.

TABLE II. DATA FROM TRANSFUSION STUDIES

CASE 1 FIG. 1, LOWER CURVES			CASE 2 FIG. 3 (BRO)			NORMAL SUBJECT FIG. 3 (HOR)			NORMAL SUBJECT FIG. 3 (RIC)			CASE 3 FIG. 4 (HEI)			NORMAL SUBJECT FIG. 4 (PJ)			NORMAL SUBJECT FIG. 4 (SPER)		
A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	2.02	0.988	1	1.95	0.750	1	5.00	0.140	1	5.45	0.211	1	1.43	0.675	1	5.55	0.217	1	5.50	0.199
3	1.89	0.885	2	1.90	0.587	2	4.95	0.150	2	5.05	0.222	2	1.33	0.733	4	5.18	0.239	4	5.75	0.233
5	2.50	1.01	3	1.77	0.638	3	5.15	0.141	5	4.95	0.200	5	2.20	0.634	5	5.40	0.215	5	5.45	0.193
7	2.30	0.855	4	1.79	0.640	6	5.00	0.117	8	5.05	0.196	6	1.56	0.627	7	5.31	0.194	8	5.00	0.192
11	2.65	0.823	7	2.06	0.709	9	5.05	0.101	14	5.55	0.203	8	1.91	0.636	11	5.51	0.182	11	5.30	0.196
14	2.60	0.762	9	2.05	0.649	15	4.90	0.088	16	4.95	0.166	12	2.80	0.526	13	5.30	0.146	13	5.15	0.202
18	2.75	0.667	10	2.11	0.790	17	4.80	0.084	22	5.10	0.147	14	2.95	0.525	15	5.45	0.167	19	5.15	0.180
25	3.00	0.562	13	2.40	0.624	24	5.15	0.078	29	4.95	0.142	16	2.80	0.555	18	5.11	0.154	22	5.25	0.173
32	2.65	0.124	16	2.65	0.563	30	4.92	0.069	34	4.95	0.130	19	3.15	0.457	20	5.31	0.146	27	0.05	0.138
33		0.067	20	2.15	0.518	33	4.68	0.078	37	4.95	0.106	21	3.25	0.515	25	5.28	0.137	32	5.15	0.149
34	2.60	0.050	23	2.75	0.513	39	4.90	0.045	41	5.05	0.098	26	3.30	0.433	28	5.75	0.125	35	5.21	0.148
35	2.60	0.027	29	2.55	0.515	45	4.90	0.047	44	5.10	0.100	30	3.55	0.427	33	5.35	0.115	39	5.35	0.139
36	2.65	0.009	38	3.20	0.413	47	4.85	0.043	48	4.85	0.094	34	3.95	0.404	36	5.45	0.105	46	5.60	0.134
39	2.65	0.000	42	3.25	0.430	51	4.68	0.037	55	4.70	0.079	40	4.15	0.346	41	5.00	0.079	53	5.50	0.133
41	2.86	0.002	44	3.75	0.385	58	4.70	0.031	58	5.10	0.074	44	3.93	0.309	46	5.30	0.098	57	5.75	0.117
1	3.55	0.356	48	3.30	0.367	65	4.75	0.030	62	4.60	0.056	48	4.45	0.278	49	5.00	0.100	62	5.40	0.092
3	3.40	0.300	50	3.58	0.338	71	4.60	0.024	65	4.90	0.066	54	4.00	0.211	53	5.95	0.085	68	5.75	0.085
6	3.40	0.078				75	5.15	0.006	69	5.30	0.053	57	4.40	0.231	57	4.95	0.083	74	5.60	0.076
7	3.55	0.015				79	4.65	0.004	71	5.20	0.050				61	5.40	0.065	78	5.35	0.055
8	3.45	0.006				86	4.80	0.002	76	4.75	0.044				64	5.50	0.065	85	5.45	0.050
9	3.35	0.002							82	5.00	0.036				82	5.70	0.048	92	5.20	0.043
10	3.25	0.000							86	4.65	0.030				88	5.40	0.043	99	5.51	0.041
									90	5.25	0.026				97	5.35	0.021	106	5.45	0.019
									98	5.15	0.016				104	5.55	0.017	116	5.70	0.002
															112	5.40	0.009	126	5.40	0.003
															119	5.05	0.007			
															127	5.05	0.003			

Column A = number of days after transfusion of tagged cells; Column B = total erythrocyte counts, 10^6/mm.^{3}; Column C = observed inagglutinable erythrocyte counts, 10^6/mm.^{3}

This adjusted value is expressed as NoQ. The NoQ value was plotted on a semilogarithmic scale on the ordinates, against time on the abscissas. A smoothed curve was drawn through the plotted NoQ values. The NoQ values were calculated from the equation: $NoQ = \frac{N}{1 - \frac{t}{T}}$ where NoQ = inagglutinable cell count corrected for the loss of cells by normal aging, N = the observed value for the inagglutinable cell count at time t, t = any day after transfusion from 0 to 120, and T = the potential lifespan of normal erythrocytes assumed to be 120 days. Assuming a T value of 100 days does not alter these curves significantly.

By this mathematical expression, curves are horizontal if there is loss only by normal aging process. Any deviation downward from the horizontal indicates loss by random destruction. If the curve is straight it represents a constant rate of loss of transfused erythrocytes.

EXPERIMENTAL STUDIES AND RESULTS

Experiment 1.—A 50-year-old woman with pernicious anemia in relapse was treated daily with injections of 15 μg of vitamin B_{12}. (Fig. 1, upper curves.) On the ninth day of therapy she was transfused with fresh normal erythrocytes of group O Rh_0 positive. The patient belongs to group B Rh_0 positive. The B_{12} injections were continued for 14 days more and discontinued (see arrow on graph, transfusion No. 1). The NoQ values show no evidence of random loss for 34 days after transfusion. Twenty days after the vitamin B_{12} was discontinued, the curve deviates from the horizontal indicating random destruction of the erythrocytes at a rate of 0.9 per cent per day. On the sixty-second day after transfusion, vitamin B_{12} injections were resumed at approximately 4-day intervals. Complete hematologic recovery took place and remission was maintained with vitamin B_{12} therapy. Several months later, while still on vitamin B_{12} injections, the second transfusion of fresh blood from the same donor was given. The NoQ values fit a straight horizontal line, indicating no random destruction. The second curve excludes the possibility that random destruction in the first transfusion was due to isosensitization.

Experiment 2.—An 80-year-old woman with severe pernicious anemia was the subject of this study. (Fig. 1, lower curves.) She had had no previous transfusions. A child of her only pregnancy was born 60 years before. She was given daily injections of vitamin B_{12} and on the fifth day of therapy, fresh donor cells of group OMN rh blood were transfused to her. The patient was of blood group B Rh_1. At this time the indirect Coombs' test using the patient's serum and donor cells was negative. For the first 30 days of this study, the NoQ values described a curve of random loss of tagged cells of about 1.3 per cent per day. This rate of loss is comparable to the rates observed in other patients with untreated pernicious anemia.[4] After the thirtieth day, there was a sudden increase in hemolysis with a loss of 50 per cent of the tagged cells per day. Treatment with B_{12} was continued and hematologic recovery from the anemia continued. A second transfusion of fresh blood from the same donor was then given. The slope of the resulting NoQ curve of inagglutinable cells indicated a 50 per cent

rate of loss of cells per day, similar to the latter part of the first curve. A direct Coombs' test, using the recipient's serum and fresh donor cells, was positive. We could not demonstrate antibodies for the antigen N in the recipient's serum. The serum of the recipient was found to contain an anti-hr′ antibody. The initial loss of red cells by random destruction in the first transfusion had a rate

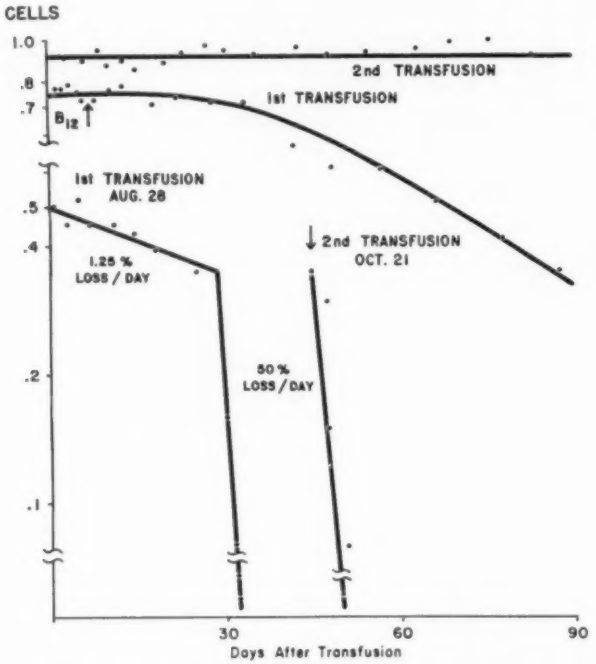

Fig. 1.—Random loss of normal erythrocytes transfused to a patient with pernicious anemia compared to random loss of normal erythrocytes due to isosensitization. The disappearance of tagged erythrocytes measured by the differential agglutination of Ashby are described by these curves. Curves which are horizontal indicate loss by normal aging whereas any downward deviation of the curve represents random destruction and the slope of the curve measures the rate of cell loss due to causes other than the normal aging process. The ordinates are plotted on logarithmic scales. In the upper two curves normal blood was first transfused to a patient with pernicious anemia who had been treated with injections of vitamin B_{12} for 9 days. On the fourteenth day after transfusion, B_{12} injections were stopped (see arrow). For about 34 days the loss of erythrocytes was by normal aging but thereafter the cells were lost at a random rate of about 0.9 per cent per day. The second transfusion, top curve, was fresh blood from the original donor transfused to this patient several months later while in remission due to continuous B_{12} therapy. The horizontal line described by the plot of the corrected inagglutinable cell indicates loss of transfused cells by normal aging only. This suggests that the random loss in the first transfusion was not due to isosensitization.

In the lower part of the chart fresh normal blood (group OMN rh) was transfused to a patient with untreated pernicious anemia (Case 1; blood group BM Rh_0). For the first 30 days the tagged cells were lost randomly at a rate of 1.25 per cent per day, similar to the rates of loss expected in untreated pernicious anemia. Around the thirtieth day there was a sudden acceleration of red cell loss to 50 per cent per day with the apparent disappearance of inagglutinable cells about 9 days later. Later, a second transfusion of fresh cells from the same donor was given to the patient, then in remission, and under continuous B_{12} therapy. The tagged cells disappeared rapidly in less than 10 days at a constant rate of about 50 per cent per day. At the end of this study anti-hr′ agglutinins were demonstrated and the indirect Coombs' test, using patient's serum and donor's erythrocytes, became positive. The rapid loss of tagged erythrocytes is due to a demonstrable immune process in distinction to the early relatively slow loss of donor cells due to the hemolysis in untreated pernicious anemia.

consistent with hemolysis due to untreated pernicious anemia whereas the rapid loss of cells in the latter part of the study strongly suggested the development of an antibody for the donor's cells.

Experiment 3.—(Fig. 2.) In Step 1, blood was withdrawn from a 79-year-old man with untreated pernicious anemia and in Step 3 his erythrocytes (group OMN Rh_0 positive) were transfused to a normal recipient of group AMN Rh_0 positive. In Step 2, blood from a normal donor (ON Rh_0 positive) was immediately transfused to the patient with pernicious anemia. The NoQ curves for the normal cells in pernicious anemia and pernicious anemia cells in a normal subject show a random loss of about 3 per cent per day.

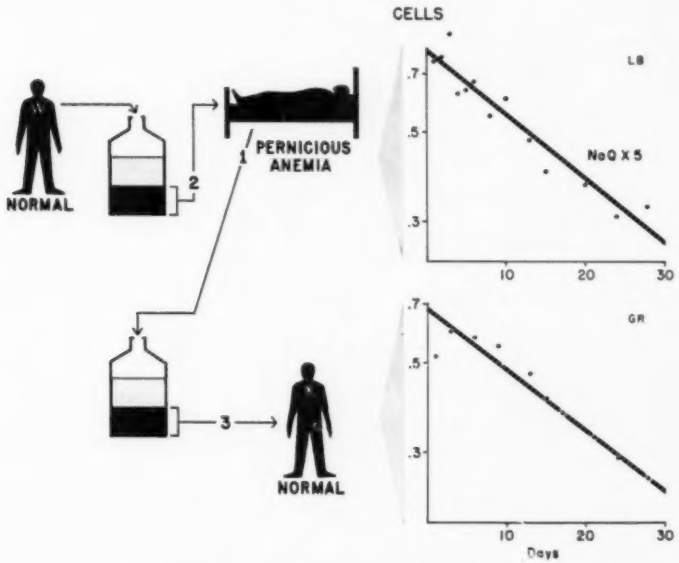

Fig. 2.—Comparison of rates of loss of transfused normal erythrocytes in a patient with untreated pernicious anemia and pernicious anemia erythrocytes in a normal person. In this study normal erythrocytes, tranfused to a person with untreated pernicious anemia, and the erythrocytes of an untreated pernicious anemia patient, transfused to a normal recipient, are lost at random in their respective recipients. The curves described by the corrected inagglutinable counts deviate from the horizontal over the 30-day period, indicating random destruction. Both rates of loss are about 3 per cent per day.

Experiment 4.—(Fig. 3.) In Step 1, blood was withdrawn from an 80-year-old man with untreated pernicious anemia. In Step 3, his cells (group OMN Rh_0 positive) were separated from the plasma and transfused to a normal recipient of group AMN Rh_1. Fresh normal erythrocytes of group OMN Rh_1rh were then suspended in the pernicious anemia plasma, stored as indicated, and, 18 hours later in Step 4, were transfused to a normal group A Rh_0 recipient. Immediately after the removal of blood from the patient with pernicious anemia, fresh blood from a normal group ON rh subject was transfused (Step 2) to the patient with pernicious anemia. The NoQ curves show that the normal inag-

glutinable cells were destroyed at random in the patient with pernicious anemia. Also, pernicious anemia cells in a normal person were rapidly lost. Likewise, the normal cells incubated in vitro for 18 hours in pernicious anemia plasma and then transfused to a normal person were hemolyzed at random. This suggests that a factor in pernicious anemia plasma injured the normal erythrocytes and caused their rapid destruction when transfused to a normal person.

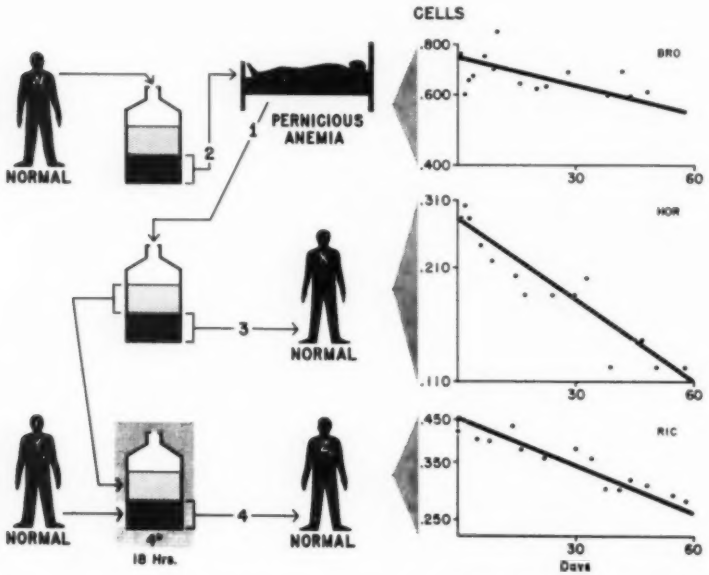

Fig. 3.——Fresh normal erythrocytes stored in plasma from a patient with untreated pernicious anemia lost by random destruction in a normal recipient. All the plotted curves of the corrected inagglutinable cell counts show deviation from the horizontal, thus indicating random loss in their respective recipients. The normal cells transfused to a patient with untreated pernicious anemia, the pernicious anemia cells transfused to a normal person, and the normal cells stored for 18 hours in the pernicious anemia plasma and subsequently transfused to a normal person were lost from circulation by random destruction. The rates of loss were considerably slower than those due to isoimmunization in the second study of Fig. 1.

Experiment 5.—(Fig. 4.) In Step 1 blood was withdrawn from a patient with untreated pernicious anemia. The pernicious anemia cells (group AMN rh) were separated from the plasma and discarded. Fresh normal erythrocytes (group ON rh) were divided into 2 portions. In Step 3 one portion was placed in pernicious anemia plasma and stored for 18 hours before transfusion to a normal recipient (group BM Rh_0 positive). The other portion of normal red cells was placed in fresh normal plasma and stored 18 hours. In Step 4 these erythrocytes were transfused to another normal recipient (group AMN rh). In Step 2 erythrocytes from a normal donor (OMN rh) were transfused to him. Note that the plasma from this normal donor was used in Step 4 for a control on the storage procedure in this study. The plot of the NoQ curve shows random destruction

of normal erythrocytes in the pernicious anemia subject. The normal cells incubated in pernicious anemia plasma and then transfused to the normal subject also showed random destruction. However, normal cells from the same donor, stored in normal plasma, survived normally in a normal subject.

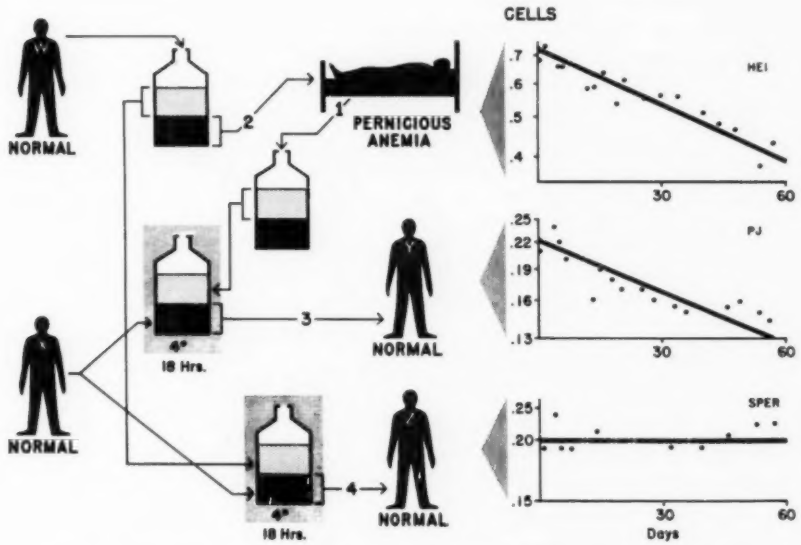

Fig. 4.—Comparison of rates of loss of fresh normal erythrocytes stored in pernicious anemia plasma and in normal plasma subsequently transfused to normal recipients. Normal erythrocytes transfused to a patient with untreated pernicious anemia and normal red cells stored in pernicious anemia plasma are destroyed at random as indicated by the slope of the plotted curves of corrected inagglutinable cell counts (upper two curves). On the other hand, normal cells stored in normal plasma and subsequently transfused to a normal recipient have a normal survival as indicated by the horizontal curve of the corrected inagglutinable cell counts.

DISCUSSION

Experiments 2, 3, 4, and 5 indicate that a hemolytic factor destroys normal erythrocytes transfused to a patient with untreated pernicious anemia. The rates of hemolysis are similar to those described previously.[4] In Experiments 4 and 5 the evidence suggests that the plama of untreated patients with pernicious anemia has a factor or a deficit capable of injuring normal erythrocytes. Normal cells so injured are destroyed at random when transfused to a normal subject. The rates of loss are similar to those of normal cells destroyed in patients with untreated pernicious anemia and of pernicious anemia cells in normal persons (Experiments 3 and 4).

It is unlikely that the manipulations of the blood in incubation were responsible for these injuries because cells from the same donor, handled in precisely the same way but incubated in normal plasma, survived normally (Experiment 5).

The exponential curves described in our studies are similar to the curve reported by Sheets and associates[6] (Case 5), where it was shown that erythrocytes from a patient with severe lead poisoning were injured in some manner so that they had a shortened survival time when transfused to a normal recipient. The NoQ curves describing the *slow* random loss of erythrocytes in these pernicious anemia studies are also similar to the rates of red cell loss in rheumatoid arthritis recently reported by Freireich and co-workers.[8] These investigators used NoQ curves in analysis of their data and described rates of loss of 0.94 to 2.39 per cent per day of normal erythrocytes transfused to persons with rheumatoid arthritis. For comparison, see Table III.

TABLE III. RATES OF RANDOM LOSS OF TAGGED CELLS IN THESE EXPERIMENTS CALCULATED FROM THE SLOPE OF THE NoQ CURVES

TRANSFUSIONS IN ORDER OF PRESENTATION (FIGS. 1 TO 4)	FIG. IN PAPER	RATE OF RANDOM LOSS OF TAGGED CELLS (EXCLUSIVE OF LOSS DUE TO NORMAL AGING) PER CENT LOSS PER DAY
1. Normal cells in pernicious anemia	1	0.9
2. Normal cells in treated pernicious anemia	1	0.0
3. Normal cells in untreated pernicious anemia; after developed antibody for tagged cell	1 1	1.25 50.0
4. Normal cells in treated pernicious anemia, loss due to acquired antibody	1	50.0
5. Normal cells in untreated pernicious anemia	2	3.0
6. Pernicious anemia cells in normal	2	3.0
7. Normal cells in untreated pernicious anemia	3	0.58
8. Pernicious anemia cells in normal	3	1.0
9. Normal cells stored in pernicious anemia plasma, then survival studied in normal	3	0.8
10. Normal cells in untreated pernicious anemia	4	0.76
11. Normal cells stored in pernicious anemia plasma, then survival studied in normal	4	0.81
12. Normal cells stored in normal plasma, then survival studied in normal	4	0.0

Our studies do not give us a clue to the nature of the destructive factor or deficit in pernicious anemia other than to speculate that the red cells are injured by contact with pernicious anemia plasma in such a way that the normal erythrocytes so treated are destroyed at random in a normal person. The results appear to exclude the function of some abnormal tissue in the pernicious anemia patient as the only cause for the destruction of the red cells. These studies do not differentiate between an acquired defect of the pernicious anemia cells and a developmental defect causing them to have a shortened survival time. In Experiment 3 the identical rates of loss of normal cells in pernicious anemia and pernicious anemia cells in normal persons suggest that the defect of the pernicious anemia cells could be accounted for on an acquired basis alone. On the other hand, in Experiment 4 the rates of loss of the pernicious anemia cells in a normal subject were slightly faster than those of normal cells in untreated pernicious anemia, suggesting the possibility of a cellular defect as well as a plasma effect. Variability of unknown biologic factors, however, may be responsible for the slight difference of rates of random loss in the two recipient subjects.

When evidence of abnormal hemolysis is obtained by use of the Ashby method of differential agglutination one is always concerned with the possibility of antibody effects. When antigen-antibody incompatibilities are discovered as causes of the hemolysis, the rates of hemolysis differentiate them from curves observed in our pernicious anemia studies. Experiment 2 is an example of an antigenic response of the recipient to donor's cells containing an Rh antigen. The first 30 days of the curve in this study showed a constant rate of loss of 1.3 per cent per day, similar to those observed in untreated pernicious anemia. Thereafter, the sudden change in rate of loss to 50 per cent per day suggested that some newly acquired extracorpuscular hemolytic mechanism was responsible. Fresh donor cells from the same donor were once more transfused into the patient and these cells were lost at a similar rapid rate. At the beginning of the study the indirect Coombs' test was negative, whereas it was positive at the end of the study. Fresh donor cells and recipient serum were used in the indirect Coombs' test. An anti-hr′ antibody was detected in the serum of the recipient at the end of the study. This is in contrast to Experiment 1 where the donor cells disappeared at a relatively *slow* rate of about 0.9 per cent per day and later fresh donor cells used in the first transfusion survived normally in the patient who had remained in remission for several months under treatment with vitamin B_{12}. Thus, in Experiment 1 the hemolytic property observed is not ascribed to blood incompatibility. Mollison,[9] drawing upon his own experience and data from other authors, has discussed the problem of blood incompatibilities. In the instance of incompatibility with demonstrable isoantibodies, the red cell disappearance curves have some of the following characteristics: The rates of loss are usually *very rapid;* the damaged or incompatible cells disappear in a few minutes, several hours, or in several days with a rapid initial loss of a large proportion of the tagged cells in a few minutes or hours followed by a slower loss. Apparently normal survival for many hours or days, followed by accelerated loss of the tagged cells may occur.

The shortened survival of some normal blood in recipients without demonstrable antibodies has been studied by Loutit, Mollison, and Young,[10] Mollison,[9] Jandl and Greenberg,[11] and Fudenberg and Allen.[21] In their studies some "compatible" bloods have been found to survive abnormally without evidence of a demonstrable antibody or other known cause. Bloods from these same donors have been transfused to other normal individuals and found to survive normally. Inspection of the published curves shows rapid rates of loss and they are similar to those due to specific isoantibodies. In one of Jandl's studies he pointed out that multiple transfusions of blood to the same recipient were successively shortened. This suggested an immune process. The characteristics of these curves are unlike those of the slow constant hemolysis observed in pernicious anemia.

It is unlikely that a single saline wash of the donor cells could damage them and lead to random loss in the recipient. The first and last transfusions of our studies show normal survival after washing the red cells once with cold 0.9 per

cent saline. Mollison and Veall[24] and Hughes Jones and Mollison[25] demonstrated that survival of red cells was not altered over the first 36 days after transfusion when cells were washed 3 to 15 times in 0.9 per cent saline in the preparation of Cr^{51}-labeled erythrocytes.

There are several studies of the survival of normal erythrocytes in persons with pernicious anemia.[1, 2, 13-16] In view of the experience accumulated by a large number of investigators studying erythrocyte survival with particular reference to methods of plotting data and obtaining mathematical analysis of the curves, Schiødt,[26] Brown,[17] Dornhorst,[18] Sheets and co-workers,[6] and DeGowin and associates,[7] it appears advisable to re-examine the original data.[1, 2, 13-16]

In 1921, Müller and Jervell[13] studied the survival of normal blood transfused into a patient with severe pernicious anemia. The inagglutinable cell counts following two transfusions of tagged cells were enumerated and plotted on Cartesian coordinates, "Kurve III." An estimate of the rates of loss of these tagged cells can be made by the NoQ method of analysis. The rate of loss of cells after the first transfusion of 1,000 $mm.^3$ of tagged cells was calculated by us to be a random loss of about 1 per cent per day for the first 31 days. Thereafter, the rate of loss suddenly accelerated so that cells were lost at a rate of about 78 per cent per day. A second transfusion of tagged cells was given. These cells were lost at a rate of 75 per cent per day. In these studies the curves are almost identical to those in our Experiment 2 where the initial slow loss of normal cells presumed to be caused by a factor in the pernicious anemia plasma was followed by a rapid loss of cells caused by isosensitization.

In 1921, Ashby transfused blood into many patients with pernicious anemia. She discussed at length the problems of attempting to obtain a true picture of cell production versus cell destruction of homologous and heterologous blood.[15] The problem still remains complicated and is by no means solved. Ashby's pioneer study should be read by anyone interested in this problem. In 3 of 4 patients studied for 90 days the survival curves were interrupted by several other transfusions. The inagglutinable counts remained high for long periods of time suggesting the possibility of changes in the potency of the antiserum. The other long-term study is of interest in that from the seventh to the eleventh day the patient started a remission. The red cell count increased from 1.6 million to 4.8 million in about 35 days. The possibility of loss of the hemolytic property during a remission must be considered. Many other cases were studied by Ashby but only for short periods, ranging from about 15 days to 30 days. They were complicated by multiple transfusions and occasionally by blood from the same donor group used for the original tagged cell study. Ashby interpreted her curves to mean that the order of destruction in limited parts of the study for donor as well as recipient cells was greater than expected and that sometimes donor cell transfusions didn't raise and sustain counts as would be anticipated. In two severely ill patients, tagged cells disappeared very rapidly over a period of a few days. She pointed out that in some of the studies there were periods of active blood destruction of both patient's red cells and donor cells, not "due to any intrinsic weakness of the corpuscle."

In 1922, Wearn, Warren, and Ames[14] transfused tagged cells to 3 patients with pernicious anemia. One study was complicated by multiple transfusions during the experiment. The few inagglutinable cell counts make interpretation of the irregular curve difficult. Another patient with pernicious anemia used in their studies was in relapse, judging by the total erythrocyte counts of the recipient. During 110 days of the study, 8 counts were above 4,000,000, 2 above 5,000,000, and 3 ranging from 3.4 to 3.6 million. The inagglutinable curve shows normal survival. The findings in a third patient are of interest for this patient was in severe relapse during the study. The first group of inagglutinable cells transfused to this patient was followed for 59 days. The authors fortunately included their experimental data in the paper. The curves obtained from the plotted NoQ values for these inagglutinable cell counts show considerable random destruction. In our analysis of this curve by the NoQ method a conservative estimate of rate of loss of inagglutinable cells between the fifth and fifty-ninth day is 1.18 per cent loss per day. The rate of loss between the thirtieth and fifty-ninth day, however, is 2.27 per cent and from the fortieth to fifty-ninth day is about 3.4 per cent loss per day. At the end of this time another transfusion was given, raising the inagglutinable cell counts and making further interpretation of the curve impossible. Thus, in re-evaluation of this curve, there is unmistakable evidence of random destruction of normal tagged cells transfused to the patient with untreated pernicious anemia.

Mollison[16] reported on the survival of normal erythrocytes in two cases of pernicious anemia. In the first patient, the curve is described by only 5 points, the last one on about the seventy-fourth day. The smooth curve through these points is a straight line with an apparent disappearance time at about 92 days. This survival is abnormal. The second case was followed for 6 days with two inagglutinable cell counts. A valid interpretation cannot be made from two observations. Mollison makes no mention of the severity of illness or clinical state of his patients in the study. Sooner or later we would expect to find normal survival of cells in "untreated pernicious anemia" for hemolytic potentials have quantitative differences and undoubtedly can be governed by spontaneous or induced remissions. In a recent red cell survival study we were unable to demonstrate a hemolytic property either to the plasma or for normal cells transfused to a lad of 17 who had severe pernicious anemia. We observed normal survival of normal cells in a patient with severe untreated pernicious anemia who had been treated with antibiotics for severe pyelonephritis and bronchitis. We do not know what effect bacterial infection and antibiotic therapy might have upon untreated pernicious anemia. Foy and Kondi[19] reported that macrocytic anemia caused by poor diet responded to oral and parenteral penicillin. We observed a significant reticulocytosis in a patient with untreated pernicious anemia who was treated with Aureomycin. Daily variation in inagglutinable cell counts may be considerable and can be accounted for in some instances on the basis of plasma volume changes of the recipient rather than from technical error.[5] It is for this reason that a large number of inagglutinable counts and simultaneously determined total erythrocyte counts should be obtained over a long period of time

in the analysis of erythrocyte survival curves. Ashby[20] noted the periodic variation in inagglutinable counts. Denstedt and associates[21] and Osborne and Denstedt[22] were concerned about the fluctuations in the inagglutinable cell count. They considered the possibility of temporary sequestration of tagged cells in the spleen and determined that the observed changes were not due entirely to experimental error but most probably had other significance.

Loutit[1] transfused normal blood stored in acid citrate glucose for 2, 5, 7, 7, and 14 days into patients with Addisonian pernicious anemia. The graphs show a 50 per cent survival of inagglutinable cells of 31, 35, 43, and 53 days. He points out that this is less than the 60 days obtained by Mollison and Young[23] for blood 0- to 4-days old in 15 recipients with hypochromic anemia. In making our analysis of these curves with the NoQ method all show loss due to random destruction. The estimated rates of random loss are 0.3, 1, 1, 1.1, and 1.9 per cent per day and are similar to those described for pernicious anemia. In their studies the short period of storage of donor blood would not produce this type of curve. In the storage of blood for 15 days or less one might expect a slight initial loss of transfused cells in the first few hours and then subsequent normal life expectancy curves of the remaining cells.

CONCLUSIONS

1. Plasma of untreated pernicious anemia in contact with normal erythrocytes so affected them that they had a shortened survival time when transfused to normal persons.

2. Normal erythrocytes are hemolyzed at random, irrespective of their ages, when transfused to patients with untreated Addisonian pernicious anemia.

3. The slow disappearance curves describing the hemolysis of tagged normal cells in patients with untreated pernicious anemia can be differentiated from the rapid curves of disappearance due to isoimmunization.

4. The erythrocyte survival curves published by other authors were reexamined using currently available methods of analysis. Several additional examples of curves showing slow random loss of normal transfused cells to patients with untreated pernicious anemia were found. The rates of loss were similar to those described in this paper for pernicious anemia.

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LABORATORY METHODS

AN IMPROVED METHOD FOR THE CHEMICAL DETERMINATION OF URINARY CATECHOL AMINES

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INTRODUCTION

THE value of the urinary catechol amine determination as an aid in the diagnosis of pheochromocytoma is becoming increasingly apparent. Several chemical methods have been employed for their determination. In general, these methods consist of adsorption of the catechol amines on alumina or aluminum hydroxide by either the column or the batch method and production of fluorescent by-products of the epinephrine and norepinephrine in the eluates. Persky[7] has written an excellent review of the earlier procedures. Modifications have recently been described by von Euler and associates,[14] Pitkänen,[8] and McMillan.[6] Unfortunately urine often contains substances which hinder its passage through a column of alumina. This difficulty was circumvented by the batch method of shaking or stirring the urine with the adsorbent.[12] The disadvantages of this technique are that the catechol amines may be subjected to increased oxidative hazards by aerating the specimen, and that the method does not ensure complete contact of the catechol amines with the adsorbent. Urine may contain substances other than epinephrine and norepinephrine that appear in the eluate, which can fluoresce under the conditions of the analyses. It is important, therefore, to be able to recognize the fluorescence due to the catechol amines alone.

The alumina column method described in this report is a modification of those of Lund,[3-5] von Euler and associates,[12, 13] and Goldenberg.[2] Two important advantages of this modification are (1) that the urine samples are prepared so that they flow rapidly through the alumina column and (2) that a better means of discriminating the fluorescences of the catechol amines is provided.

MATERIALS AND METHODS

Reagents.—

Aluminum oxide: Woelm, nonalkaline, grade 1.* Fine particles were removed by repeated suspension in distilled water and decantation. It was reactivated by heating at 400° C. for 3 hours.

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*Obtainable from Alupharm Chemical Company, 322 Lafayette Street, New Orleans, La.

Sodium acetate (0.2 M, pH 8.5): Dissolve 16.4 Gm. of anhydrous sodium acetate, reagent grade, in double glass-distilled water to make 1 liter. This was adjusted to pH 8.5 with saturated aqueous sodium carbonate.

Sodium carbonate: Twenty-five per cent w/v.

Sulfuric acid: Accurately titrated, 0.25 N.

Acetate buffer (1 M, pH 6.3): Add 4 ml. 1 M acetic acid and 0.8 ml. of 2.5 N NaOH to 96 ml. 1 M sodium acetate. When 1 ml. of this solution is mixed with 0.2 ml. 0.1 N H_2SO_4 the pH should be 6.0.

Ascorbic acid: Two per cent aqueous w/v. Prepare shortly before use.

Sodium ascorbate: Mix 9 parts of 5 N NaOH with 1 part of 2 per cent ascorbic acid within 1 to 2 minutes of use.

Distilled water: Double glass-distilled or "for parenteral use."

Standard solutions: Stock standard epinephrine solutions (0.1 per cent) were prepared by dissolving 100 mg. crystalline epinephrine (free base, Parke-Davis) or 181.9 mg. of epinephrine bitartrate (Sterling-Winthrop* or Winthrop-Stearns) in 100 ml. 0.1 N H_2SO_4. The 0.1 per cent epinephrine solutions available in ampules (Parke-Davis) are suitable but decompose readily on opening. Stock standard norepinephrine solutions (0.1 per cent) were prepared by dissolving 20.1 mg. crystalline *l*-arterenol bitartrate (Winthrop-Stearns or Sterling-Winthrop*) in 10 ml. 0.1 N H_2SO_4. Working standard solutions are 1:1,000 dilutions of the stock in 0.1 N H_2SO_4. Stock standard epinephrine has been kept for 6 months in the refrigerator without change. Working standards deteriorate and should be prepared weekly even though we have found some stable for longer periods when kept at refrigerator temperatures. A dilute sodium fluorescein solution (1:1,000 dilution of 0.0005 per cent) in 0.1 N sodium hydroxide was used routinely to check the fluorescence of the working standards.

Preparation of the alumina columns: To 2.5 Gm. of alumina was added 5 ml. of sodium acetate (pH 8.5) and the stirred slurry was washed into chromatographic columns (14 mm. by 30 cm.) equipped with a stopcock.† Prepare as many columns as there are aliquots.

Treatment of Urine Specimens.—Twenty-four-hour urine samples were collected in brown bottles containing 10 ml. of 6 N HCl and 2 Gm. oxalic acid crystals. The specimens were refrigerated during and following collection. Urine volumes were measured and the urines with a small volume were adjusted to 1,500 ml. with distilled water. Sufficient urine is filtered through Whatman No. 40 paper to give 150 ml. filtrate. This is adjusted to pH 1.5 to 1.8 with 6 N HCl and divided into three 50 ml. aliquots in Erlenmeyer flasks. One of these may be used to determine recovery in which case 1 ml. of a 0.001 per cent norepinephrine solution may be added to provide a 20 μg per cent addition. The aliquots are heated in a boiling water bath for 15 minutes and cooled under running tap water. To each cooled urine specimen is added 30 ml. of acetate buffer (pH 8.5) and further adjusted to pH 8.5 by small additions of 25 per cent Na_2CO_3. It is immediately poured on the column and allowed to pass as rapidly as possible. The columns are not allowed to run dry. The alumina is washed first with 10 ml. of acetate buffer (pH 8.5) and then with 50 ml. of glass-distilled water. H_2SO_4 (0.25 N) is added and allowed to pass at a decreased speed. When the eluate becomes acid to bromthymol blue paper, 10 ml. is collected. This represents a fivefold concentration of the original material. The eluate is now analyzed for fluorescent substances. Eluates have been kept at 2° to 5° C. for 4 months without change.

Fluorometric Analyses.—The treatment of the unknowns and standards is exactly the same in the production and analyses of the fluorescent substances. A blank for each standard and unknown is provided and carried through the same procedures except in the timing and sequence of addition of ascorbic acid and sodium hydroxide. The fluorescences were

*This material was furnished by M. L. Tainter, M.D., Director of Sterling-Winthrop Research Institute.

†Obtainable from Microchemical Specialties Co., University Avenue, Berkeley, Calif.

measured with a Farrand photofluorometer equipped with the following filters: (primary) Corning Nos. 5860, 5113, and 3389 and (secondary) Corning Nos. 3384 and 5433. (The No. 3389 filter, however, has been found unnecessary as will be shown.)

Two-tenths milliliter (0.2 μg) of each working standard and 0.2 ml. of each eluate are placed in a duplicate series of 15 ml. glass-stoppered graduated tubes. One tube of each is treated as the blank and the other as the sample tube. To all of the blank tubes is added 1 ml. of acetate buffer, pH 6.3, and 0.1 ml. 0.25 per cent potassium ferricyanide. Timing is started, the tubes are shaken and allowed to react 2 minutes. Nine-tenths milliliter of 20 per cent NaOH is added, mixed, and allowed to stand 10 minutes. This process is now repeated with the sample tubes except that after the ferricyanide treatment, 1 ml. of very freshly prepared sodium ascorbate is added. One-tenth milliliter of 2 per cent ascorbic acid is now added to the blanks. The timer is set for 10 minutes. Meanwhile double-distilled water is added to make 10 ml. in all tubes. These are inverted for mixing and aliquots are transferred to respective cuvettes. At the end of the last 10-minute period the fluorescences are compared. Timing is an important feature of the foregoing phase and must be adhered to. Sixteen tubes is a maximum number to handle. This represents the samples and blanks of the 2 standards and 6 eluates.

Three series of readings were made using 3 primary filter systems providing activating wave lengths at 365 mμ, approximately 400 mμ, and 436 mμ. One secondary filter system (5433 and 3384) passing a narrow band at 510 mμ was used to measure emitted light. The epinephrine standard was set in all cases to read 50 and its blank zero. All other blanks and sample tubes including those for norepinephrine were compared to this.

Calculations.—Using 0.2 ml. of eluate representing a fivefold concentration of the original urine and 0.2 ml. (containing 0.2 μg) of the standards, the following calculations apply:

$$\frac{\text{(Eluate sample minus eluate blank)}}{\text{(Nor. sample minus nor. blank)}} \times 20 = \mu\text{g per cent total catechol amines as norepinephrine}$$

$$\mu\text{g per cent} \times \frac{\text{(24 hr. vol. or 1,500)}}{100} = \mu\text{g per 24 hours.}$$

These values are determined for each wave length reading. The values obtained with the 365 mμ and 400 mμ filters appear to be more specific. This fact was first determined when water solutions of known norepinephrine concentrations, and later urine analyses, were carried through the entire procedure.

RESULTS

To determine the precision of the method, 10 aliquots of the same urine specimen were analyzed. Using the fluorescence value obtained with the 5860 primary filter, the catechol amine content (as norepinephrine) averaged 12.18 μg per cent, ± S.D. 1.4, range 10.0 to 14.0. Using the fluorescent value obtained with the 5113 primary filter, the average was 11.6 μg per cent ± S.D. 1.12, range 9.5 to 13.1. The readings with the 436 mμ exciting light averaged 18.2 μg per cent. The latter value is in agreement with our experience with 42 specimens where values were obtained with the 436 mμ light which did not agree with the values obtained from readings at the 2 other wave lengths. When dilutions of the eluates were made, comparable reductions in fluorometer readings were obtained.

In Table I are the data for per cent recovery of norepinephrine when added to urine. It is seen that when 20 μg per cent is added, the recovery averages 63 per cent, and when 200 μg per cent is added, the recovery averages 75 per cent.

TABLE I. RECOVERY OF NOREPINEPHRINE ADDED TO URINE

μG PER CENT NOREPINEPHRINE ADDED	NUMBER OF SPECIMENS	RANGE OF RECOVERY (PER CENT)	AV. PER CENT RECOVERY
200	6	54 to 100	75
20	19	30 to 100	63

Norepinephrine was added in varying concentrations to a "synthetic urine." This consisted of 100 ml. of M/15 KH_2PO_4, 100 ml. of M/15 Na_2HPO_4, 10 ml. of 6 N HCl, and 2 Gm. of acid oxalic crystals (reagent) with water to make 1 liter. Fifty milliliters aliquots of the above solution containing known amounts of norepinephrine were treated exactly as urine in the procedure. Ten sets of 4 concentrations were run. The results listed in Table II show an average recovery of 81 per cent. Standard deviations decreased with increased concentrations used. The results shown were taken from the 365 mμ primary filter readings.

TABLE II. PERCENTAGE RECOVERY OF NOREPINEPHRINE FROM PHOSPHATE-BUFFERED WATER

EXPERIMENT NO.	MICROGRAMS PER CENT ADDED			
	NONE	10	20	30
1	0.5	92	75	63
2	0.0	91	91	71
3	2.0	74	76	75
4	0.0	65	71	70
5	1.0	74	74	77
6	0.0	94	88	92
7	0.0	65	77	98
8	0.0	83	86	94
9	3.0	76	80	74
10	0.6	97	91	91
Average	0.7 μg	81%	81%	81%
Standard deviation		±11.8%	± 7.6%	± 7.1%

Range 63 to 98 per cent recovery.
All quantities calculated from readings with the 365 mμ primary filter.

TABLE III. FREQUENCY VARIATION OF TOTAL CATECHOL AMINES IN THE URINES OF 42 MEN AND WOMEN

μG PER CENT	NO. SPECIMENS	μG PER 24 HR.	NO. SPECIMENS
0 to 4	3	0 to 70	2
5 to 9	17	71 to 140	15
10 to 14	15	141 to 210	13
15 to 19	4	211 to 280	9
20 and above	3	281 to 350	3

The urines of 42 adults were analyzed in duplicate and the data are presented in Table III. Twenty-four-hour excretion levels were found to range between 0 and 342 μg as norepinephrine. The average was 169 μg ± S.D. 73. The frequency variation is also presented in Table III. There was a standard deviation of ± 2.17 between these duplicates. These urines were from normotensives and hypertensives without demonstrated pheochromocytoma.

The normal level of excretion of catechol amines by this method is considerably higher than has been reported by many others. A comparison is shown in Table IV.

TABLE IV. COMPARISON OF TOTAL EXCRETION OF CATECHOL AMINES (AS NOREPINEPHRINE) IN NORMAL URINE AS REPORTED BY VARIOUS INVESTIGATORS

INVESTIGATOR	METHOD OF ISOLATION	NORMAL RANGE
Goldenberg et al.[2]	Extraction with $Al(OH)_3$	7 to 100 μg per 24 hr.
McMillan[6]	Al_2O_3 batch	5 to 190 μg per 24 hr. > 1 μg per 24 hr. as epinephrine
Pitkänen[8]	Al_2O_3 batch	0 to 100 μg per 24 hr.
von Euler et al.[13]	Al_2O_3 batch	0 to 100 μg per 24 hr.
Zeleli[15]	Al_2O_3 batch	20 to 50 μg per 24 hr.
Sobel and Henry[11]	Al_2O_3 column	0 to 14 μg per 100 ml.
Proposed method	Al_2O_3 column	0 to 22 μg per 100 ml. 0 to 315 μg per 24 hr.

DISCUSSION

The advantages of the proposed method are the incorporation of a rapid column isolation procedure with a fluorometric analysis which permits increased reliability of the result. Adding oxalic acid to the collection bottle solved the vexing precipitate problem. Urine can now be filtered free of the material at an acid pH where the catechol amines are relatively stable. Its rapid adsorption and elution are ensured. Fig. 1 shows the pH conditions to use for proper concentration of the eluted catechol amines in the eluate. Under the conditions provided in the fluorometry of these substances, norepinephrine has been made to exhibit a change in fluorescent intensities dependent upon the activating wave length (Fig. 2). Most urine eluates and eluates obtained from aqueous norepinephrine solutions put through the column respond to 2 of these wave lengths (365 mμ and 400 mμ) as does the norepinephrine standard. This suggests that norepinephrine is the principal catechol amine of most urines and corroborates McMillan's[6] findings. Analysis of Fig. 2 shows that under the conditions of the test pure norepinephrine exhibits a 365:400 mμ ratio of 1.7 ± 0.1, but, when diluted with epinephrine, the same ratio approaches 1.0. Tetracyclines from urine fluoresce in an area close to norepinephrine. One urine containing a tetracycline exhibited a 365:400 mμ ratio of 1.3. Our considered judgment at this time indicates that variations of this ratio beyond the limits of 1.5 to 1.9 for an eluate is proper evidence of contaminating fluorescence which would invalidate the results.

The normal values found by this method are substantially higher than have been reported by other investigators (Table IV). The author feels this is due in part to oxalic acid. Aside from its ability to clean up the urine, it has been shown[10] to protect epinephrine under oxidizing conditions. It may also chelate copper and iron in the specimen and eliminate their catalytic

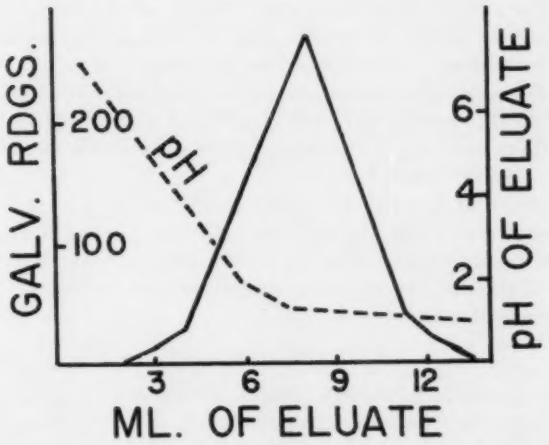

Fig. 1.—Fractional elution of the alumina column following the adsorption of urine containing 100 μg per cent added norepinephrine. The galvanometer readings represent the fluorescent material in each successive milliliter of eluate after 0.25 N H_2SO_4 was added to the column. pH measurements were taken of each fraction for correlation.

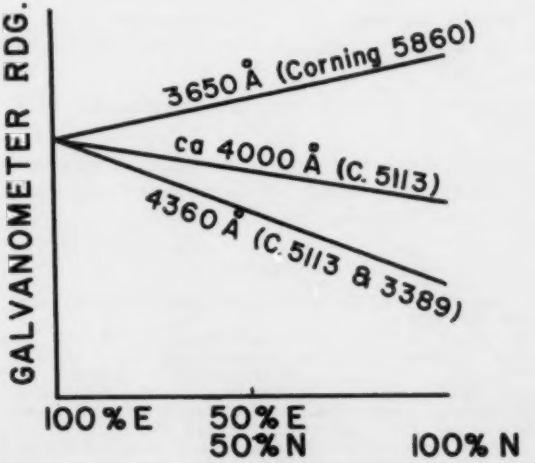

Fig. 2.—Fluorescence of solutions having the same total catechol amine concentration but varying in per cent epinephrine and norepinephrine. The fluorescences (represented by galvanometer readings) were determined following the ferri-cyanide-alkali treatment and referred to pure epinephrine arbitrarily set to 50 units. The influence of the activating wave length to distinguish norepinephrine is demonstrated. Emitted light was measured by secondary Corning filters Nos. 5433 and 3384 passing 510 mμ.

oxidation of the catechol amines. Interference from the fluorescence of ascorbic acid is eliminated in the method. With the fivefold concentration, eluate readings are found to range near those of the standards. The important[13] control of pH has been taken care of in the oxidative step to develop fluorescent by-products. The eluates routinely have an acidity equivalent to 0.1 N acid. By using this acid strength in the standards and adjustment of the buffer to compensate for it, a pH of 6.0 ± 0.1 can be expected throughout all tubes. This avoids tedious titration, a process in which the catechol amines may be inadvertently destroyed.

The specificity of Lund's[5] fluorometric determination of epinephrine and norepinephrine from which the proposed method is modified has been discussed in detail elsewhere.[9] Whether or not the methoxy derivatives of the pressor amines, recently[1] shown to be important metabolites, are contributing to the values reported here is unknown.

SUMMARY

1. A method has been presented which allows large quantities of urine to be rapidly chromatographed on alumina columns. The addition of oxalic acid with the hydrochloric acid provides uniformly "clean" specimens.

2. Alterations in the method of Lund[5] and of von Euler and Floding[12, 13] are introduced which reduce extraneous fluorescence and standardize the procedure.

3. An increased specificity in the fluorescence analysis is obtained. Two exciting wave lengths are used and the fluorescences of the standard norepinephrine solution; the eluates are compared against the fluorescence of standard epinephrine.

4. The method has a reproducibility of approximately ± 10 per cent in the normal range.

5. Total catechol amine excretion as norepinephrine averages 169 μg per 24 hours ± S.D. 73 by this method. Norepinephrine appears to be the major component of the active amines in urine.

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DETERMINATION OF MAGNESIUM IN ERYTHROCYTES

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MAGNESIUM is an important component of intracellular fluids and is second only to potassium as the major intracellular cation. About 98 per cent of total body magnesium is intracellular where it is involved as a cofactor in many enzymatic processes.

The extracellular fluid (serum) level of magnesium has been measured in various disease states. Low serum magnesium values have been found in patients who showed clinical signs of hallucinations, delirium, and a characteristic athetoid or flapping tremor.[1, 2] These clinical manifestations were relieved by parenteral magnesium administration. However, some patients with similar clinical states and symptoms have shown normal serum magnesium concentrations but were also relieved by magnesium therapy. Conversely, patients with asymptomatic hypomagnesemia have also been observed. It has been postulated that the intracellular magnesium level may be of more importance in reflecting body deficit and in determining symptomatology.

In order to study intracellular magnesium in erythrocytes a method using whole blood has been developed. The red blood cell was chosen because it is easily obtained from patients. Erythrocyte magnesium has been previously reported by Orange and Rhein[3] in a limited number of normals using the titan yellow method. However, since extracellular fluid magnesium and calcium are measured in our laboratory by the method of Zak and associates[4] a modification of this method has been developed for determination of erythrocyte magnesium.

MATERIALS AND METHODS

Reagents.—(1) Reagents as in determination of serum magnesium and calcium.[4] (2) Twenty per cent trichloroacetic acid (TCA). (3) Twenty-five per cent potassium thiocyanate. (4) Isoamyl alcohol.

Equipment.—Equipment as in determination of serum magnesium and calcium.[4]

Analytical Procedure.—The procedure consists of measurement of total divalent cations and calcium in heparinized whole blood and plasma. From these values and the hematocrit, erythrocyte magnesium is calculated.

Whole Blood Magnesium and Calcium.—Pipette 1 part of thoroughly mixed blood into a mortar. Layer 2 parts of distilled water onto the surface of the blood and rinse the pipette with the clear supernatant. Add another 2 parts of distilled water; repeat the rinsing and

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mix. Add 1 part of trichloroacetic acid; grind thoroughly to an homogenous suspension. Transfer material to a centrifuge tube and allow it to stand for 5 minutes. Centrifuge for 5 minutes and filter the supernatant through ashless filter paper. Removal of iron is then accomplished by adding 1 part of potassium thiocyanate to 3 parts of the whole blood filtrate and extracting the iron (red) complex with 2 parts of isoamyl alcohol. The isoamyl alcohol extraction is then repeated.

Three milliliters aliquots of the iron-free filtrate, equivalent to 0.375 ml. of whole blood, are used in the determinations. To one aliquot, add 3 ml. of 0.4 N sodium hydroxide and 0.25 ml. of murexide indicator. To another aliquot, 3 ml. of ammoniacal buffer and 0.20 ml. of eriochrome black T indicator are added. The spectrophotometric titrations of calcium and total divalent cations are then performed as described by Zak and associates.[4]

Plasma Magnesium and Calcium: One part of plasma is treated with 4 parts of distilled water and 1 part of TCA, centrifuged and filtered. Three milliliters aliquots, equivalent to 0.50 ml. of plasma, are used to determine calcium and total divalent cations as described for whole blood.

Blanks: Distilled water is used as the blank for the standard solutions. A filtered mixture of distilled water and TCA is used for the plasma blank. The same mixture, after addition of potassium thiocyanate solution and extraction with isoamyl alcohol, is used for the whole blood blank. Blank values must be subtracted from the amounts of reagents used in the corresponding titrations.

RESULTS

Recoveries.—The recovery data for calcium and magnesium from known mixtures of these cations and iron are shown in Table I. It may be seen that the recoveries are almost as accurate as with mixtures of calcium and magnesium alone which are shown in Table II.

TABLE I. RECOVERIES OF CALCIUM AND MAGNESIUM FROM KNOWN MIXTURES CONTAINING ABOUT 1 MG. PER 100 ML. OF FERRIC CATION (MEQ./L.)

CALCIUM			MAGNESIUM			TOTAL DIVALENT CATIONS		
PRESENT	FOUND	AVERAGE	PRESENT	FOUND	AVERAGE	PRESENT	FOUND	AVERAGE
3.70	3.58	3.67	3.50	3.86	3.78	7.20	7.45	7.45
3.70	3.75	(99%)	3.50	3.70	(108%)	7.20	7.45	(103%)
2.60	2.54	2.54	3.50	3.73	3.73	6.10	6.27	6.27
2.60	2.54	(98%)	3.50	3.73	(107%)	6.10	6.27	(103%)

TABLE II. RECOVERIES OF CALCIUM AND MAGNESIUM FROM KNOWN MIXTURES (MEQ./L.)

CALCIUM			MAGNESIUM			TOTAL DIVALENT CATIONS		
PRESENT	FOUND	AVERAGE	PRESENT	FOUND	AVERAGE	PRESENT	FOUND	AVERAGE
5.00	5.00	5.09	2.00	2.08	1.92	7.00	7.08	7.01
5.00	5.22	(102%)	2.00	1.72	(96%)	7.00	6.94	(100%)
4.00	3.83	3.83	3.29	3.42	3.41	7.29	7.26	7.24
4.00	3.83	(96%)	3.29	3.39	(104%)	7.29	7.23	(99%)
5.00	5.00	4.97	2.00	1.92	1.97	7.00	6.92	6.94
5.00	4.94	(99%)	2.00	2.03	(99%)	7.00	6.96	(99%)

Normal Men.—Determinations of plasma and erythrocyte magnesium have been done on 13 normal men (Table III). Plasma magnesium ranged from 1.55 to 2.06 mEq./L. with a mean of 1.80 ± (S.D.) 0.13 mEq./L. Erythrocyte magnesium ranged from 4.69 to 5.86 mEq./L. with a mean of 5.29 ± 0.34 mEq./L. The R.B.C./plasma magnesium ratio ranged from 2.38 to 3.48 with a mean of 2.95 ± 0.32.

TABLE III. MAGNESIUM VALUES IN NORMALS (MEQ./L.)

NO.	ERYTHROCYTES	PLASMA	R.B.C. / PLASMA
1	4.69	1.91	2.46
2	4.90	2.06	2.38
3	5.36	1.80	2.98
4	5.86	1.79	3.27
5	4.98	1.84	2.70
6	5.41	1.55	3.48
7	4.84	1.64	2.95
8	5.80	1.73	3.35
9	5.19	1.76	2.95
10	5.32	1.99	2.68
11	5.53	1.87	2.95
12	5.43	1.72	3.16
13	5.46	1.76	3.10
Mean ± S.D.	5.29 ± 0.34	1.80 ± 0.13	2.95 ± 0.32

DISCUSSION

Red blood cell magnesium has been studied previously by Orange and Rhein[3] who used the titan yellow method. They studied 5 normal subjects and found a range of 4.85 to 5.59 mEq./L. with a mean of 5.25 mEq./L. These results are quite similar to those in the present study.

The spectrophotometric titration of calcium and magnesium with ethylenediaminetetraacetic acid (EDTA) has proved quite useful for serum analysis.[4] This method shows a high degree of reproducibility and has the distinct advantage of measuring both calcium and magnesium. Our data indicate that a modification of this method may be utilized to determine erythrocyte magnesium. Since iron forms a chelate with EDTA, it is necessary to remove this cation from whole blood filtrates prior to titration. This is accomplished by combining the iron with potassium thiocyanate and extracting this red complex with isoamyl alcohol. Comparative recovery studies indicate that this iron-removal procedure does not interfere with the accuracy of the subsequent titration.

TCA was selected for the removal of blood proteins to avoid the addition of cations present in many precipitating agents. It has the further advantage of producing the acid pH necessary for the subsequent extraction of iron. Since the protein-bound magnesium is released by TCA,[3] one must treat the plasma as well as the whole blood with this reagent to have comparable values.

The possibility that the addition of the several reagents to precipitate protein and remove iron might affect the EDTA titration of calcium and magnesium was considered. The recovery data indicate that the effect of these reagents on the titration may be corrected if proper blank solutions are run concurrently and subtracted from the amount of reagent used for the samples.

SUMMARY

A procedure for determination of magnesium in erythrocytes, using a modification of the Zak method for serum calcium and magnesium, has been described.

Recovery studies proved satisfactory.

The range for erythrocyte magnesium in 13 normal men is 4.69 to 5.86 mEq./L. with a mean of 5.29 mEq./L. (S.D. ± 0.34 mEq./L.). The R.B.C./plasma magnesium ratio in these normals ranged from 2.38 to 3.48 with a mean of 2.95 ± 0.32.

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ACTINOMYCES BOVIS AND "ANAEROBIC DIPHTHEROIDS": PATHOGENICITY FOR HAMSTERS AND SOME OTHER DIFFERENTIATING CHARACTERISTICS

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ACTINOMYCES bovis and some strains of "anaerobic diphtheroids"* are sufficiently similar in morphology and growth habits to create a problem in differentiation.

Although, in general, small laboratory animals are resistant to infection with *A. bovis*, a preliminary study[1] suggested that the young male golden hamster might serve to demonstrate pathogenicity of *A. bovis*, thus providing an important point of differentiation from anaerobic diphtheroids. Of 8 pure isolates of *A. bovis*, all induced infection after a single peritoneal injection of saline suspensions of the unwashed sediments of the broth cultures. The induced lesions revealed certain features of clinical actinomycosis, including granules and clubs. However, too few isolates were studied to justify definite conclusions; furthermore, factors which might have artificially enhanced invasiveness, such as injection of the meat-infusion broth in the unwashed inocula, were not controlled. Nevertheless, the results were sufficiently promising to warrant further experiments, including studies of the comparative pathogenicity of *A. bovis* and anaerobic diphtheroids for the young hamster. Included in the studies were colonial and microscopic morphology of the microorganisms cultured in both solid and liquid media, and also observations of some enzymatic activities, in order to determine whether or not a correlation existed between these characteristics and pathogenicity.

MATERIALS AND METHODS

Fifteen pure strains of *A. bovis* were studied as well as an equal number of isolates of anaerobic diphtheroids, all of which were recovered from pathologic material. Attempts to obtain species of Actinomyces of bovine origin with cultural characters of those described by Erikson[2] and Thompson[3] or of *Actinomyces naeslundi*[4] were unfortunately unsuccessful. Of the combined total of 30 isolates of *A. bovis* and anaerobic diphtheroids, 11 were recovered in this laboratory from cultures of materials streaked directly in series on 3 Petri plates containing beef-heart infusion agar with horse blood (F43.A)[5a] or after preliminary culturing in beef-infusion broth (F5A)[5b] with 1 per cent glucose. For the other isolates we are indebted to the late Dr. Rhoda Benham of Columbia University; Dr. Esther Meyer, University of Illinois College of Medicine; Dr. John Slack, University of West Virginia; and to the American Type Culture Collection, Washington, D. C.

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*Use of the generally accepted term "anaerobic diphtheroids" avoids generic classification of a group of microorganisms whose importance lies only in their superficial similarity to certain pathogens.

The isolates have been maintained for 1 to 4 years, at first only on slants of beef-heart infusion blood agar and more recently on this medium alternating with brain-heart infusion agar for revitalization purposes. They are usually subcultured at 4-week intervals at which time the transplants are incubated at 35° to 37° C. for 10 days to 2 weeks under anaerobic conditions and then stored in the refrigerator at 3° to 6° C. For anaerobic cultivation the Fildes-McIntosh jar is used unless otherwise indicated. Anaerobiosis is controlled by an indicator.[5c]

For determination of the effect of oxygen, duplicate Petri plates of beef-heart infusion blood agar were inoculated with each one of the 30 isolates. One set was incubated aerobically at 35° to 37° C. and the other anaerobically. The plates were inspected at 7 and 14 days.

The appearance of the colonies and microscopic morphology in stained films prepared from the isolates after cultivation on the beef-heart infusion blood agar were studied as were stained films of the isolates grown in beef-infusion glucose broth and thioglycollate broth. The growth characteristics of the isolates on Sabouraud's glucose agar (F57.1:B)[5d] were also investigated.

To demonstrate certain enzymatic activities the isolates were grown in: skim milk with bromcresol purple as indicator (F18:B)[5e]; beef-extract gelatin (F31),[5f] and Thiogel[6]; and a casitone-yeast extract medium containing salicin, xylose, and raffinose. The last-named medium is prepared as follows.*

> Combine five grams of Bacto yeast extract, 15 Gm. of Bacto casitone, 2.5 Gm. of sodium chloride, 0.75 Gm. of agar, 400 ml. of water, and 100 ml. of 0.04 per cent bromthymol blue; adjust pH to 7.2; dispense material in 5 ml. amounts in Durham tubes and autoclave; add singly in 5 ml. amounts a 2 per cent solution of the carbohydrates salicin, xylose, and raffinose to give a final concentration of 1 per cent.

The inocula for the casitone-yeast extract medium were prepared from washed sediments of broth cultures resuspended in 0.85 per cent salt solution and adjusted visually to approximately equal opacities with the saline solution. Two drops of the suspension was inoculated to each tube of the medium. The cultures were then incubated anaerobically at 35° to 37° C. and any evidence of acid and gas formation was noted after 1, 2, and 4 weeks. For determination of the presence of catalase the test recommended by Suter[7] was followed.

Pathogenicity tests entailed the use of male golden hamsters 3 to 4 weeks of age. Most of the animals were bred at the Laboratory Farm maintained by this Division. The suspensions of cultures were prepared for injection as previously reported[1] except that the broth cultures were incubated from 10 to 14 days instead of 7 days. The cultures, incubated in duplicate, were centrifuged; the sediments were then combined and washed 2 or 3 times in relatively large amounts of sterile 0.85 per cent salt solution. The entire washed sediment was made up to 2 ml. in sterile salt solution. The suspensions of washed sediment in the case of *A. bovis* contained many large aggregates which were ruptured by repeatedly drawing up and expelling the material with a syringe until it could be easily injected through a 22 gauge needle. In spite of this treatment, the suspensions remained granular or floccular in contrast with the suspensions of anaerobic diphtheroids which were always homogeneous. One milliliter of the well-mixed suspension was injected intraperitoneally into each of two or more hamsters. The animals were observed for 4 weeks for palpable lesions, then sacrificed and examined for gross evidence of infection. Pus from abscesses was examined microscopically for actinomycotic granules and for branching filaments in films stained by Gram's method. Cultures were also made.

*For the formula we are indebted to Mr. J. Dennis Pollack, Department of Dermatology, Columbia University, New York, N. Y.

TABLE I. THIRTY ISOLATES OF *A. bovis* AND ANAEROBIC DIPHTHEROIDS

NUMBER	SOURCE
43395	Pus from abscess, neck, and shoulder
50155	Bloody exudate, mass on thigh
5105	Sputum
5272	Bloody exudate, lesion lower jaw
5273	Pus from sinus in neck
53162	Pus from cheek
53163	Cervicofacial
5542	A.T.C.C. No. 10048
5557	External ulcer, lower jaw
5561	Pus from cheek
5562	Cervicofacial
55145	Brain abscess
55146	Cervicofacial
55243	Lumpy jaw
55244	Lumpy jaw
55245	Wrist bone lesion
5603	Trochanteric abscess
5604	Blood culture
5648	Human case of actinomycosis
5652	Bovine actinomycosis
5649	Human case of actinomycosis
5650	Human case of actinomycosis
5743	Blood culture
5744	Blood culture
5745	Eye
5746	Pulmonary actinomycosis
5747	Right parotid region
5749	Subcutaneous abscess
38411	Pus
57129	Intraoral incision

TABLE II. SUMMARY OF DIFFERENTIAL CHARACTERS OF 30 ISOLATES OF *A. bovis* AND ANAEROBIC DIPHTHEROIDS

CRITERIA	*A. bovis* (15 ISOLATES)	ANAEROBIC DIPHTHEROIDS (15 ISOLATES)
A. Pathogenicity for hamster	Positive	Negative
B. Growth on:		
1. Beef-heart infusion agar with horse blood (2 weeks)	Colony: White, raised, dry, rough surface, and irregular margin; closely adherent to medium; removable intact; tough and difficult to emulsify	Colony: White, soft, low convex; smooth surface and entire margin; not adherent; soft and easily emulsifiable
	Morphology: True branching filaments, V and Y forms and rod forms	Morphology: No branching filaments; pleomorphic bacillary forms
2. Sabouraud's glucose agar (2 weeks)	No growth	Moderate soft growth
3. Beef-infusion broth plus 1 per cent glucose (2 weeks)	Clear broth; sediment flocular to granular; growth of fine colonies up sides of tubes not unusual	Faint to moderate turbidity; white, compact, heavy sediment; white confluent column of growth adhering to wall of tube
C. Effect on gelatin (2 to 4 weeks)	No liquefaction	Liquefaction
D. Effect on skim milk with indicator (2 to 4 weeks)	0 to ± acid; no coagulation	Acid and coagulation
E. Fermentation reactions (2 to 4 weeks)		
Raffinose	Acid; no gas	Negative
Salicin	Acid; no gas	Negative
Xylose	Acid; no gas	Negative
F. Catalase reaction	Negative	Positive

RESULTS

The types of cases from which the 30 strains of *A. bovis* and anaerobic diphtheroids were obtained are given in Table I.

The cultural and biochemical characteristics are summarized in Table II. All of the isolates proved to be either microaerophilic or anaerobic. The 15 strains of *A. bovis* cultured on beef-heart infusion blood agar produced white, raised, dry colonies with rough surfaces and irregular margins, closely adherent to the medium, removable intact, and difficult to emulsify (Fig. 1, *A* and *B*).

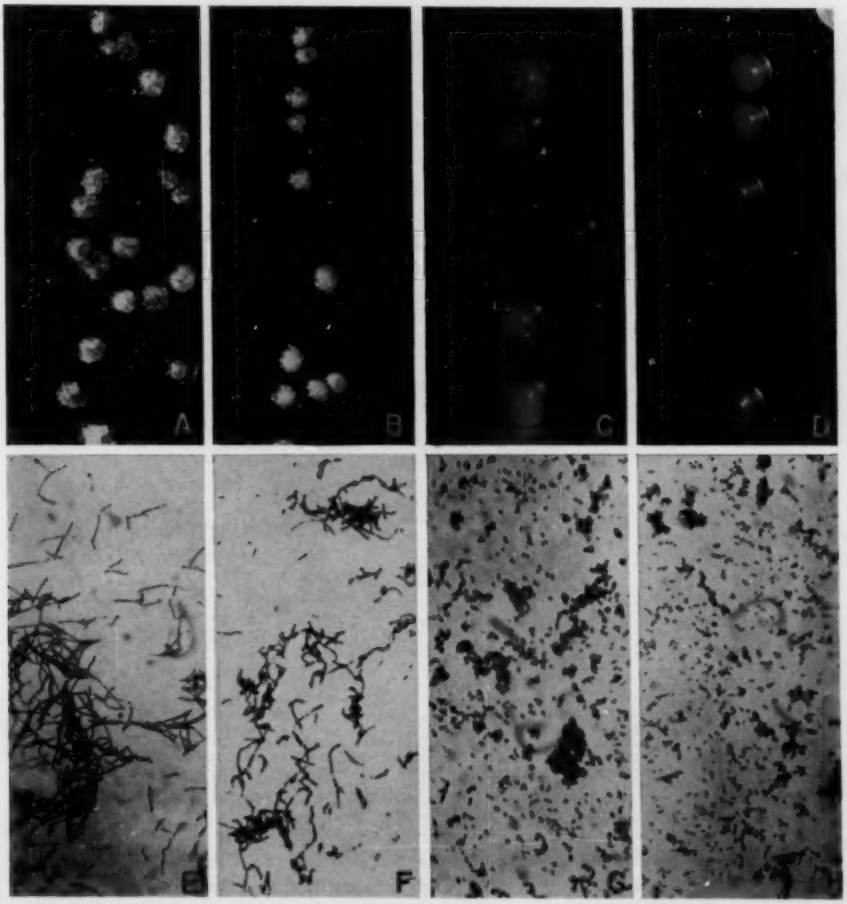

Fig. 1.—Representative colonies of *A. bovis* and anaerobic diphtheroids and their microscopic morphology. A, and B, *A. bovis* (isolates Nos. 43395 and 50155) on blood agar after 2 weeks' incubation under anaerobic conditions (magnification ×18; reduced 5/11). C and D, Anaerobic diphtheroids (isolates Nos. 5744 and 5745) on blood agar after 2 weeks' incubation under anaerobic conditions (magnification ×18; reduced 5/11). E and F, Stained film preparations from colonies A and B (magnification ×780; reduced 5/11). G and H, Stained film preparations from colonies C and D (magnification ×780; reduced 5/11).

The colonies of the anaerobic diphtheroids, on the other hand, were white, low, convex, moist, with smooth surfaces and entire margins, not adherent to the medium, soft, and easily emulsifiable (Fig. 1, *C* and *D*). In films of the colonies stained by Gram's method, *A. bovis* appeared as gram-positive, true branching filaments (Fig. 1, *E* and *F*) while the anaerobic diphtheroids showed short, gram-positive unbranched bacillary forms exhibiting considerable pleomorphism (Fig. 1, *G* and *H*). None of the isolates of *A. bovis* grew on Sabouraud's glucose agar, confirming the observation of Skinner, Emmons, and Tsuchiya.[9] On the other hand, all of the anaerobic diphtheroids produced a moderate amount of soft growth.

The appearance of beef-infusion broth cultures of *A. bovis* was characterized by a clear supernatant and a flocccular or granular sediment, usually with a growth of fine colonies extending up the sides of the tubes. The anaerobic diphtheroids produced a faint to moderate turbidity of the broth and a white, compact, smooth, heavy sediment, with a white confluent column of growth adherent to the wall of the tube. In the thioglycollate broth there was greater similarity in growth characteristics among some members of these two groups than in the beef-infusion broth. The microscopic appearance of the isolates grown in the liquid medium did not differ essentially from those cultivated on the solid medium.

TABLE III. AUTOPSY FINDINGS IN HAMSTERS SACRIFICED 4 WEEKS FOLLOWING A SINGLE INJECTION OF WASHED CULTURES OF *A. bovis*

CULTURE NUMBER	NUMBER OF ANIMALS INJECTED	NUMBER OF ANIMALS INJECTED	REACTION*
43395†	2	1	Severe and extensive
50155†	2	1	Severe
5105†	2	1	Severe
5272†	2	2	Severe and extensive
5273†	2	2	1 severe and extensive; 1 severe
5562	2	1	Severe and extensive
55162	2	2	Severe
53163	2	2	1 severe and extensive; 1 mild
57129	4	2	Mild
5561	2	2	Severe
55146	6	6	4 severe; 2 mild
5542	2	2	1 severe and extensive; 1 severe
55145	2	2	1 severe; 1 mild
5557	2	2	1 severe and extensive; 1 severe
5749	4	3	Mild

*Severe and extensive reaction: Multiple encapsulated abscesses of from 1 to 15 mm. in diameter throughout the abdominal cavity; the abscesses contained purulent material of soft or cheesy consistency and showed the presence of granules with clubs. The infection extended through the parietal peritoneum and abdominal wall or through the diaphragm with a resultant sinus. Severe reaction: Essentially the same as above but infection did not extend beyond abdominal cavity. Mild reaction: Small encapsulated abscesses, single or multiple, containing purulent material in which granules with clubs were demonstrated.

†Unwashed sediments of broth culture tested previously.

A. bovis produced slight (±) or no change in the reaction of skim milk and no coagulation; the anaerobic diphtheroids acidified and coagulated the milk. Peptonization of milk by the microaerophilic diphtheroids is employed by Ajello[8] as a factor in differentiating them from *A. bovis*. The isolates of *A. bovis* did not liquefy gelatin while the anaerobic diphtheroids did. *A. bovis* produced

acid but no gas in the liquid media containing raffinose, salicin, and xylose, respectively, whereas none of the anaerobic diphtheroids utilized these carbohydrates. These findings are in accord with those of King and Meyer.[6] *A. bovis* did not yield a catalase reaction, but the anaerobic diphtheroids did so. The results are in agreement with those of Suter,[7] and of King and Meyer.[6]

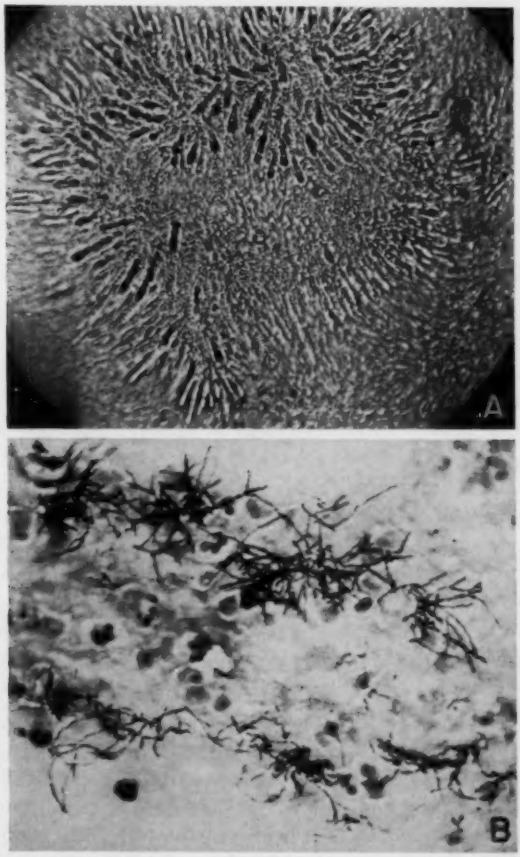

Fig. 2.—A, Unstained granule with clubs in pus from encapsulated abscess in hamster injected with *A. bovis* No. 5561 (magnification ×780; reduced 5/11). B, Stained film preparation of pus from abscess from same hamster (magnification ×780; reduced 5/11).

At least 2 hamsters (more than 2 in a few instances) received single intraperitoneal injections of the washed sediment of broth cultures of each of the 15 strains of *A. bovis*. As indicated in Table III, 31 (81.3 per cent) of the total of 38 hamsters injected showed gross evidence of infection upon autopsy 4 weeks later. In 22 the reactions were severe; there was extensive abscess formation

throughout the abdominal cavity with adhesions involving the liver, diaphragm, stomach, omentum, mesentery, spleen, kidney, and peritoneal wall. In 7 of the animals the infection had extended from the peritoneal cavity either through the abdominal wall or through the diaphragm, forming sinus tracts. Three strains induced a severe infection in one or more of the inoculated hamsters but only a mild infection in the others of the same group. Six strains caused extensive disease in one or more hamsters, but no gross lesions in others of the same group. In all, 4 developed mild lesions and 7 (17.9 per cent) of the 38 hamsters no demonstrable lesions.

Microscopic granules with clubs (Fig. 2, *A*) were found in the fresh pus from every animal with encapsulated abscesses; tangled masses of branching filaments and diphtheroid fragments were found in stained films of the pus (Fig. 2, *B*). *A. bovis* was isolated from the pus from every infected animal.

The anaerobic diphtheroids proved to be nonpathogenic for hamsters.

DISCUSSION

The discussion relates to the pathogenicity of *A. bovis* for small laboratory animals. Many attempts to reproduce actinomycosis in animals with features resembling the clinical disease in man have usually met with failure. Single or repeated injections of pure cultures of *A. bovis*, employing various routes and techniques of inoculation for enhancement of invasiveness, have not consistently induced features of the natural disease including granules and clubs so characteristic of this type of infection. The extensive earlier literature on this subject has been reviewed by Rosebury.[10] More recently Meyer and Verges[11] developed a mouse pathogenicity test as a diagnostic aid in identification of *A. bovis*. They found that intraperitoneal injection of a mixture of *A. bovis* and hog gastric mucin in albino mice resulted in progressive actinomycosis in 94.6 per cent of the animals, and in only a few instances did the same method enhance the invasiveness of anaerobic diphtheroids sufficiently to induce lesions. In these instances the lesions differed from those induced by *A. bovis*.

In our experiments a high percentage of young male golden hamsters injected intraperitoneally with a single dose of concentrated saline suspensions of washed sediments of broth cultures representing 15 strains of *A. bovis* showed evidence of infection with features of clinical actinomycosis, including granules and clubs. The suspicion that the beef-heart infusion broth in the inocula used in the previous pathogenicity tests[1] mght have intensified the invasiveness of *A. bovis* does not seem to have been justified. Indeed as shown in Table III, 5 isolates used in the previous study were retested after being washed in relatively large amounts of sterile salt solution and were found to induce the same type of reactions as did the unwashed inocula.

The marked variation in the response of duplicate hamsters to equivalent doses of 4 of the 15 isolates suggests differences in susceptibility of individual hamsters to infection by *A. bovis*. The limited data also indicate that some

strains of *A. bovis* may lack pathogenicity for the golden hamster since 2 strains induced either mild infections or no demonstrable lesions. Further work is necessary before a definite conclusion can be reached on these points.

None of the 15 isolates of anaerobic diphtheroids induced infection in the hamster. These results are essentially in agreement with those of Meyer and Verges[11] and Suter[7] based on their work with mice. Beerens,[12] in pathogenicity tests using, among other animals, hamsters, found an alleged strain of *A. bovis** to be without invasiveness for hamsters. This strain was found by us to have the morphologic and cultural characters of the anaerobic diphtheroids and is included among the representatives of this group under No. 38411.†

Thus, pathogenicity tests in hamsters represent a useful addition to the morphologic and biochemical studies used to differentiate *A. bovis* and anaerobic diphtheroids.

CONCLUSION

The young male golden hamster‡ is a suitable test animal for the determination of pathogenicity of *A. bovis*.

A. bovis injected intraperitoneally into the young male golden hamster induces infection in a high percentage of the animals. The infection has certain features of clinical actinomycosis including granules with clubs.

Anaerobic diphtheroids, injected intraperitoneally into the hamster, do not cause infection.

Colonial and microscopic morphology and certain enzymatic activities of *A. bovis* and anaerobic diphtheroids have been correlated with their pathogenicity for the hamster.

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*American Type Culture Collection No. 8374.

†This strain (No. 38411) and another (No. 38410) listed as *A. bovis* 8374 and 8373 in *Catalog of the American Type Culture Collection*, ed. 5, 1949, have the following history: They were received by the Division of Laboratories and Research, New York State Department of Health, numbered 38411 and 38410 in September, 1938, from the National Institute of Health, Washington, D. C., and labeled 9937 (human) and 9932 (bovine), respectively. In 1942, the two strains were sent to the A.T.C.C.; in 1955, Dr. Freeman A. Weiss, curator of the A.T.C.C., called attention to the publication by H. Beerens (Ann. Inst. Pasteur **84**: 1026, 1953) and stated that A.T.C.C. 8374, 8373 are *Corynebacterium acnes* or perhaps *C. liquefaciens*. Independently, in 1952, we studied these strains under numbers 38411 and 38410 and found them to have the morphologic and cultural characters of the so-called "anaerobic diphtheroids." It seems possible that the original cultures may have been contaminated with an anaerobic diphtheroid which finally supplanted *A. bovis*.[6, 12]

‡The results of a limited number of tests with young male white hamsters 3 to 4 weeks of age suggest that they are less susceptible to infection with *A. bovis* than young male golden hamsters.

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A SAFE HIGH-SPEED BLENDOR FOR PROCESSING INFECTIOUS MATERIALS

M. R. Hilleman, Ph.D., and R. O. Taylor
Washington, D. C.

IN SPITE of its indispensable function in most virus laboratories, the high-speed blendor remains a prime hazard to the laboratory worker because of occasional leakage or aerosolization of the infectious material.[1-5] Ideally, the blendor should be safe, efficient in triturating tissue, nondestructive of the infectious agent, and economical in terms of initial cost and upkeep; it should be easily decontaminated, cleaned, and prepared for further use. For maximal safety, the blendor should be sealed during operation, should allow no escape of contents between bearing and shaft or through faulty gaskets, and a means should be provided for removal of the contents without opening the bowl. To obviate degradation by heating, of the infectivity of the material being processed, the bearings should be outside the blendor bowl. Finally, all materials used for construction which come into contact with the bowl content should be corrosion-proof and essentially inert biologically. None of the blendors available commercially or described experimentally[4, 5] present all of these qualities considered essential. A high-speed blendor which combines these features has been developed in this laboratory and has proved highly effective for the processing of infectious disease agents. The present communication describes the essential features of the apparatus. Detailed construction drawings are available upon request from this laboratory.

MATERIALS AND METHODS

Constructional Details.—The present blendor bowl is a modification of the standard or semimicro jar supplied by several manufacturers.* Fig. 1 presents photographs of the modified standard and semimicro bowls and Fig. 2 shows the detailed plan of construction. All parts, unless otherwise stated, are constructed from free-machining stainless steel (Type 303). The blending assembly, with which the bowl is equipped by the manufacturer, is removed and certain of its parts are utilized for the construction of the new assembly. The lid is machined from stainless steel and screws to a brass ring which is threaded and silver-soldered to the top of the bowl. Sealing is provided by a 3/16 in. rubber washer in the lid. The bowl is fitted with 1/4 in. air inlet and drain tubes, and the hole in the bottom of the bowl, which accommodates the original blending assembly as supplied, is plugged with a stainless steel rivet and silver-soldered. The modified new blendor assembly consists of a 3/8 in. diameter driven shaft which is positioned by 2 Oilite bearings (3/4 in. outside diameter) mounted in a machined stainless steel housing. The socket head from the original blending assembly is used to engage the motor stud, and the original cutting blade is also utilized. The spray deflector is machined from stainless steel and is silver-soldered to the driven

From the Department of Respiratory Diseases and the Division of Instrumentation, Walter Reed Army Institute of Research, Washington, D. C.
Received for publication Dec. 12, 1957.
*Among them, the A. H. Thomas Co., Philadelphia, Pa., cat. nos. 4282-D7 and 4282-F.

shaft. The bearing housing is fastened, on the threaded end, to the lid using the stainless steel hexagonal nut from the original assembly. The cup for dry ice is fashioned from 3 in. brass tubing and flat sheet brass stock.

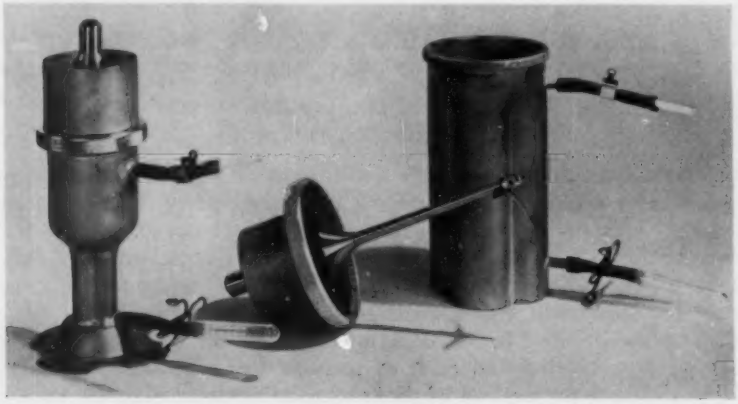

Fig. 1.

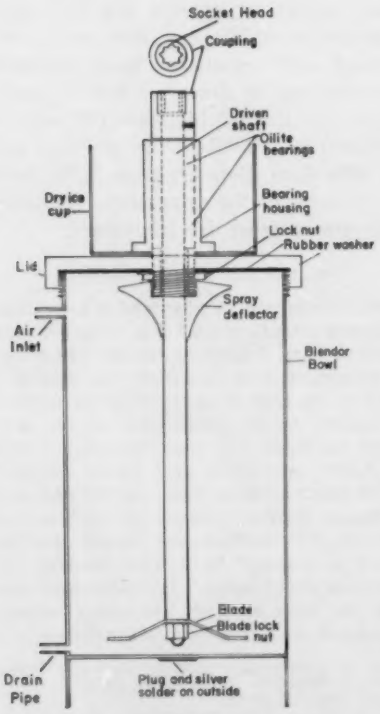

Fig. 2.

The drive unit, shown in Fig. 3, consists of an 18,000 r.p.m., 0.5 h.p. continuous operation Dumore motor* which is fitted with a ¼ in. square drive stud and machined brass guide, and is mounted on an upright stand. The blendor bowl rests on a platform which is raised or lowered by a sliding brass wedge and which is held in position by a spring-activated locking device. For use with the semimicro bowl, a 1¼ in. thick spaceblock is inserted on top of the platform. The motor speed is controlled by means of a variable transformer (Powerstat Type 116).

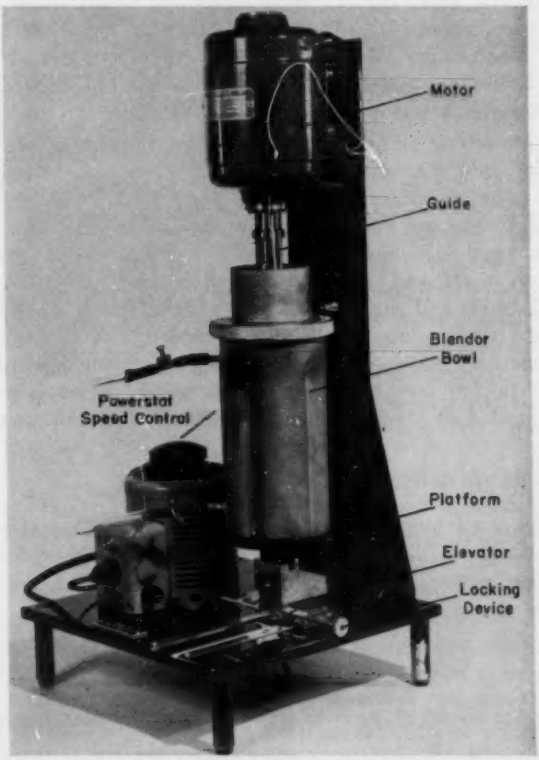

Fig. 3.

Operation.—The air inlet of the bowl is fitted with a cotton-plugged air filter and the drain pipe is equipped with a drain tube and protective glass cover. These are further protected by a paper wrapper. After introducing a few drops of water, the bowl is sealed and sterilized in the autoclave. During use, the air inlet and drain tubes are clamped off. The material to be blended is added aseptically to the bowl and the lid is screwed down tightly. The blendor bowl is engaged with the motor and operated at the desired speed for the required length of time. A dial setting of 85 on the Powerstat gives approximately 10,000 r.p.m., and 3 minutes at this speed are employed routinely. For runs longer than 3 minutes, dry ice may be added to the ice cup to facilitate cooling. To empty the blendor, the clamp is removed from the air inlet and the protective tube is removed from the drain

*The Dumore Co., Racine, Wis.

tube. The drain tube is then inserted aseptically into the sterile receptacle, the clamp removed, and the contents drained out. The bowl is decontaminated by autoclaving prior to washing and preparing for reuse.

DISCUSSION

The blendor described has proved highly effective in triturating virus-infected materials in this Institute. The apparatus was found to be safe and simple in operation requiring only a minimum of maintenance. The cost of construction was reasonable. The charge in this Institute for labor and materials for constructing 36 standard bowls was about $78.00 each and for 36 semimicro bowls was about $71.00 each. The cost for labor and materials to build the motor unit was estimated to be $219.00.

During autoclaving of the oil-lubricated bowls, a very small amount of oil was distilled into the bowl itself. While of no consequence in ordinary operations, it was considered important to prevent oil contamination of materials used to prepare vaccines for human injection. This problem was solved by treating the Oilite bearings with benzene to remove oil followed by coating of both shaft and bearings with Drilube.* This is composed of molybdenum disulfide and various resins, and provides a dry, solid, surface film lubricant. The Drilube plated assemblies withstood 8 to 12 hours' continuous operation before wearing out as evidenced by chattering and binding. This represents 160 to 240 normal 3-minute runs which, when viewed from this aspect, was regarded as highly efficient. When the lubricant is worn out, the shafts and bearings must be replated.

SUMMARY

A new high-speed blendor which is simple, economical, and safe for processing infectious and other materials in the laboratory has been described. Details for construction and operation are presented and the efficiency in ordinary use is discussed.

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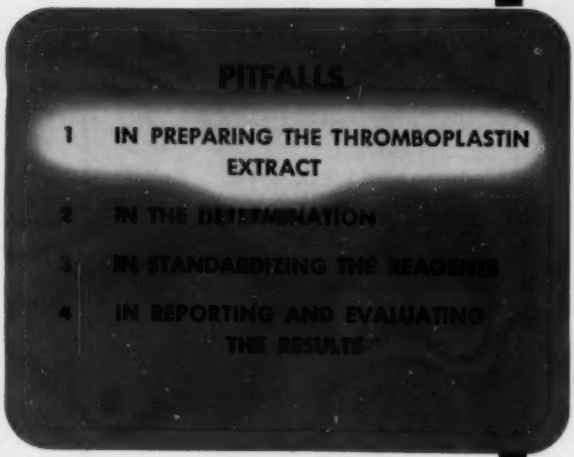
*Drilube, 723 W. Broadway, Glendale, Calif.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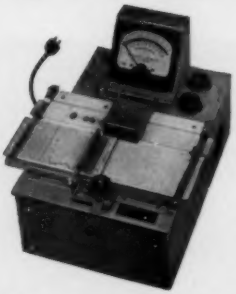

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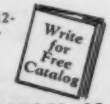

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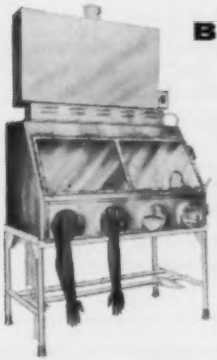

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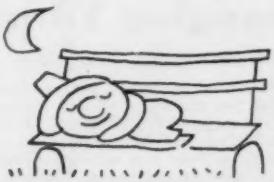

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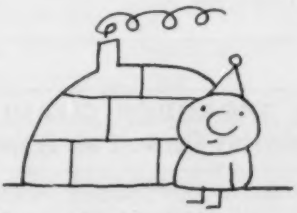

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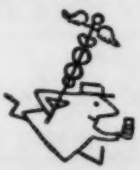

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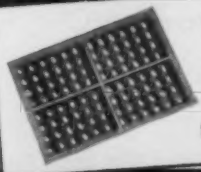

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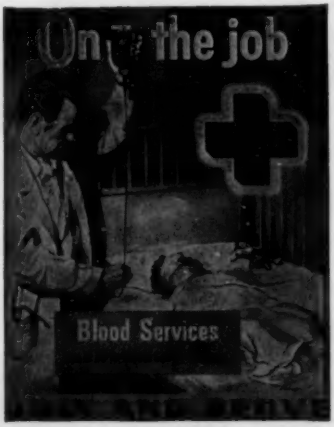

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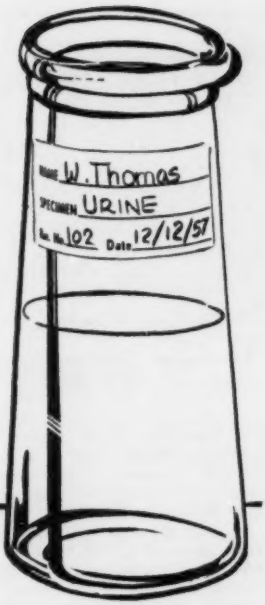

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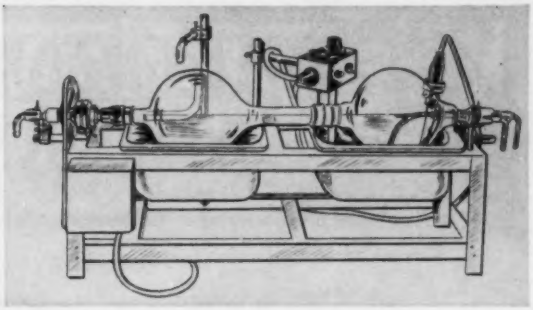

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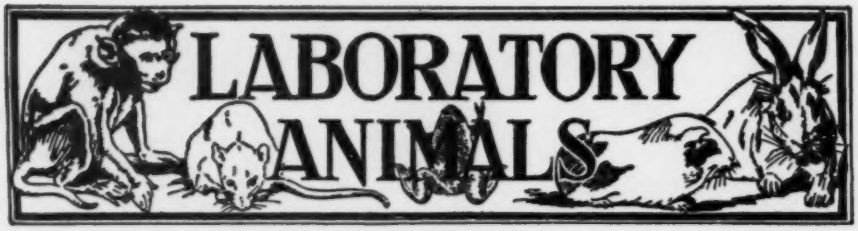

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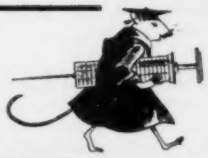

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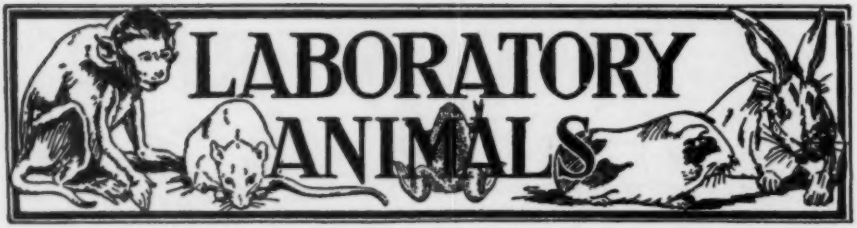

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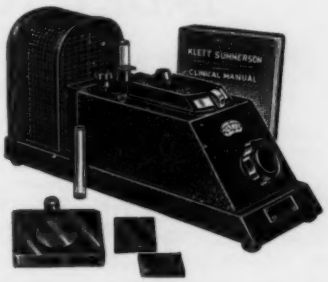

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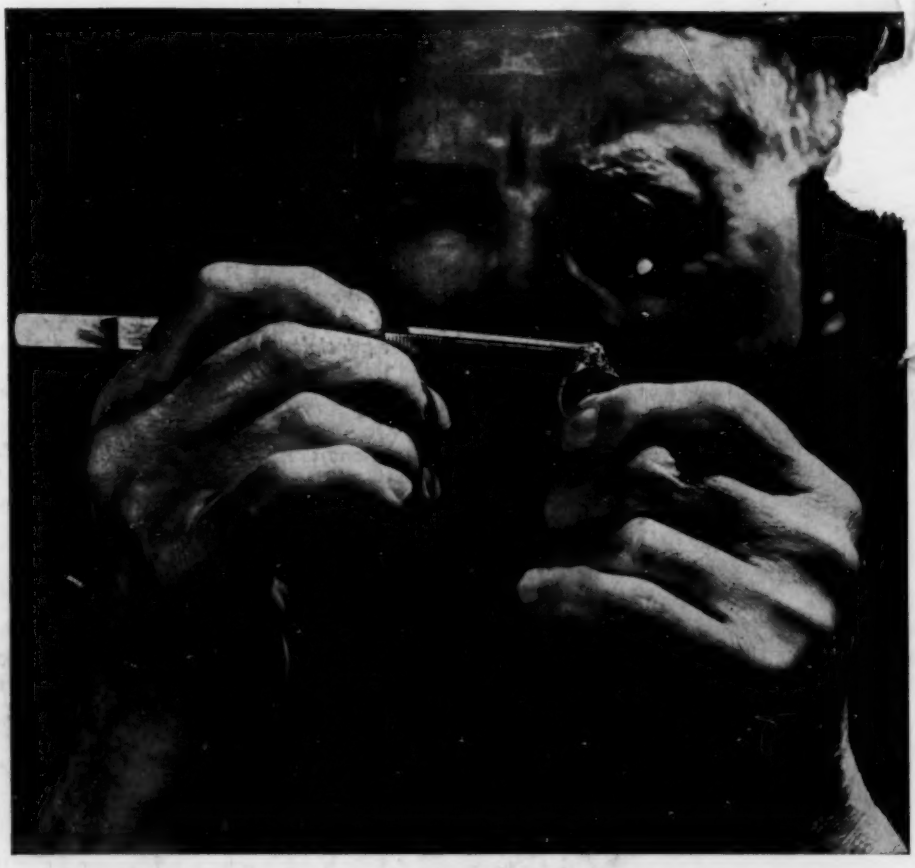

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